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Principles of Species Adjustment
I. Continuous Exposure

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INTRODUCTION

That there is a similarity between the process known as carcinogenesis and the process by which small, single-celled, free-living organisms become adjusted to unfavorable environments has been suggested by us in a series of papers (*1, 2, 3, 4, 5*). These studies were concerned with the mechanism of species adaptation of small organisms to carcinogenic agents and led us to the further suggestion that a cancer cell might be considered as a normal cell that has been able in some way to make a more or less successful adjustment when exposed to an unusual or unfavorable environment over a period of time embracing several or many cell-division cycles. The adjustment is successful only from the standpoint of the cancer cell itself. It is, of course, fatal from the standpoint of the host. It was also pointed out that the numerous clinical examples of a steplike derivation of malignant growth, the often very definite association of cancer with chronic irritation and chronic inflammation, the prolonged incubation period in experimentally induced cancer, as well as the various precancerous lesions described by clinicians and pathologists, all seemed to fit in with the concept of gradualism as a reasonable hypothetical mechanism in the genesis of cancer.

In the early reports from the Imperial Cancer Research Fund, Bashford noted "the association of cancer with peculiar and very different forms of irritation." He commented "that, as data increase from year to year, the mediate relation between irritants and cancer in particular sites of the body becomes more and more significant."

Recently, Murphy (*6*) has stated that the experimental results in the study of cancer offer definite support to the following conclusions:

(1) Malignancy is a universal cell potentiality in that any cell has inherent in its make-up the potentiality for unlimited or uncontrolled growth.

(2) The degree of this potentiality for malignancy is a variable quantity for each tissue or cell type, and this degree is determined largely, if not entirely, by hereditary or predisposing factors.

(3) The maglignancy potentiality of a cell may be developed in the more sensitive groups by the strain of normal physiologic processes but may be set off even in resistant groups by a variety of inciting agents.

556830—43— 1

(4) The change from a normal to a malignant cell represents an alteration in the cell itself, by virtue of which proliferation becomes an automatic process independent of a continuously acting provocative agent.

(5) The new property of the cell appears to develop suddenly, and this becomes a fixed character which is transmitted to all its descendants. It may possibly be the result of a somatic mutation.

It should be noted that although "the new property of the cell appears to develop suddenly," this in no way excludes the view that the sudden change may be the culmination of, and the first manifestation as a result of a series of subliminal stimuli which have extended through many cell divisions or cell generations. Inasmuch as the number of physical and chemical agents found to induce cancers in experimental animals is increasing rapidly, it seems now well-established that there is no single inciting cause of cancer, and that "malignancy is a universal cell potentiality," at least in mammals.

Principle of Continuous Exposure

For the past 4 years our experiments have been concerned with species adaptation of small organisms to carcinogenic agents in the belief, as stated in our first paper (*1, p. 129*), that—

> a systematic study of the action of carcinogenic agents upon free-living, single-cell, and small multicellular forms will ultimately throw much light upon the mode of action of these same agents upon mammalian tissues.

At the present writing (April 1943) observations upon three species of bacteria (*Eberthella typhosa, Escherichia coli,* and *Streptococcus hemolyticus*), a paramecium (*Paramecium multimicronucleatum*), and a flat worm (*Stenostoma tenuicaudatum*) have yielded data in repeated tests supporting what we have chosen to call the principle of continuous exposure. This principle, which at present has been found to be applicable to a few species only, may be stated briefly thus:

The continuous exposure of a species to a harsh or unfavorable environment may not be fatal to individual organisms for a number of generations or cell-division cycles, but in due time all individuals of the species will die.

MATERIALS AND METHODS

Although these methods have been described before, they are again outlined in part here for convenience and clarity (*5, p. 2*):

Into 15-ml.-capacity pyrex-glass vaccine vials are placed 5 or 6 ml. of plain sugar-free broth. Each vial is fitted with a rubber stopper which has a central depression or moat with a thin diaphragm at the bottom of the moat for the ready penetration of a hypodermic needle. The rubber stoppers are loosely fitted into place with a short cotton thread between the stopper and the mouth of the vial. Without this precaution the stoppers will be blown out when autoclaved at 15 pounds pressure for one-half hour. When removed from the autoclave and still warm, the cotton threads are withdrawn and the stoppers tightly fitted. After cooling, there is usually a slightly negative pressure in each vial. This helps to preserve an air-tight seal. The vials are then incubated at 37° C. for 7 days. In this way we have prepared about a thousand vials of sterile broth, and no contaminations have occurred.

One-milliliter-capacity tuberculin syringes with 26-gage needles are employed for the inoculation and transfer of bacteria from vial to vial. The small-gage needles prevent the thin rubber diaphragm from being torn. The syringes and needles are cleaned with tap water and 95 percent alcohol, thoroughly dried, placed in large test tubes with cotton stoppers, and autoclaved between usages.

This method of serial transfer is believed to be exceptionally free from the possibility of airborne contaminations and appears to be equally as safe as our pyrex-glass-box technique described elsewhere [*(7)*]. In fact, the method was tested by setting up 2 series of 100 vials each

containing sterile broth, and each day for 100 consecutive days one-half milliliter of broth was transferred by means of the sterile syringes from 1 vial to the next in series. After 7 days' incubation, no vials of either series displayed any growths. In addition, a single vial was penetrated each day for 30 consecutive days and one-half milliliter of broth was withdrawn completely and re-injected into the vial. In this case no precaution was taken to swab the stopper with tincture of iodine which is our custom when cultures are transferred. No growth appeared in this vial after the 30-day period and after 7 days' further incubation although the broth was capable of growing *Escherichia coli* with which it was finally inoculated.

Figures 1 and 2 demonstrate the relative freedom of our technique from the chance of air-borne contaminations.

RESULTS

Results With Bacteria

Figure 3 displays our tests in which attempts were made to make daily serial transfers of three different species of bacteria by incubating all cultures at a temperature considerably higher than the optimum. It may be seen that there were 15 separate series of tests, 3 on *Eberthella typhosa,* 9 on *Escherichia coli,* and 3 on *Streptococcus hemolyticus.*

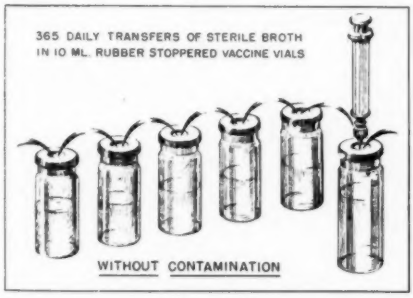

FIGURE 1.

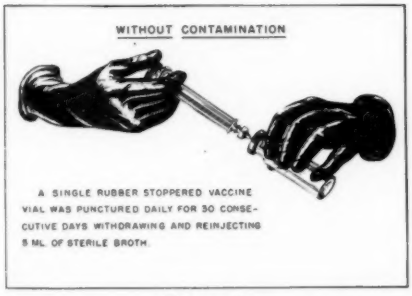
WITHOUT CONTAMINATION
A SINGLE RUBBER STOPPERED VACCINE VIAL WAS PUNCTURED DAILY FOR 30 CONSECUTIVE DAYS WITHDRAWING AND REINJECTING 5 ML. OF STERILE BROTH.

FIGURE 2.

In making the daily transfers, each fresh sterile vial was first incubated for 20 minutes at a high temperature before receiving one-half milliliter of inoculum from the previous vial in the series in order to make certain that the continuously multiplying species would never experience a temperature below that selected for any given test. After 24 hours' incubation at the high temperature, and after the inoculation of the next vial in the series, each vial was then incubated at 37° C. for 2 or 3 days and finally stored at room temperature.

Of the three typhoid series (A, B, and C) incubated continuously at 46° C., the disparity as to the day of death will be noted. The A series died on the thirteenth day of transfer, while the B and C series died on the twenty-sixth and twenty-seventh days, respectively. An even greater disparity is noted in the three tests on streptococci which were incubated continuously at 42°. The B series died on the thirty-ninth day while the A and C series died very early, the third and fourth days, respectively.

In the case of the nine tests on *Escherichia coli,* death time decreased as the tem-

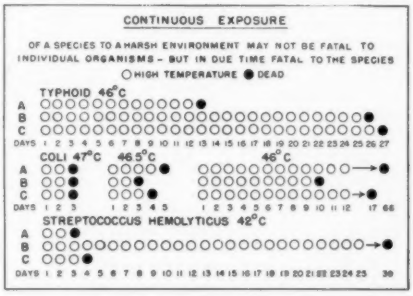
CONTINUOUS EXPOSURE
OF A SPECIES TO A HARSH ENVIRONMENT MAY NOT BE FATAL TO INDIVIDUAL ORGANISMS - BUT IN DUE TIME FATAL TO THE SPECIES
○ HIGH TEMPERATURE ● DEAD
TYPHOID 46°C
A
B
C
DAYS 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
COLI 47°C
46.5°C
46°C
A
B
C
DAYS 1 2 3
1 2 3 4 5
1 2 3 4 5 6 7 8 9 10 11 12 17 66
STREPTOCOCCUS HEMOLYTICUS 42°C
A
B
C
DAYS 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 30

FIGURE 3.

perature at which the species was kept increased. At 47° C., all three series died on the third day. At 46°, however, the A series survived 65 days, the B series only 9 days, and the C series 16 days.

The A, B, and C series of each organism were always planted from the same culture, at the same time with the same amount of inoculum (0.5 ml.). At present, we offer no explanation for the differences in the observed survival times.

The visible amount of turbidity in the last surviving vials was just as great after 48 hours as in the first vials of the series, although a perceptible slowing up of growth as roughly measured by visible turbidity was apparent in the last few vials of the series during the first 24 hours' incubation.

These tests are relatively easy to carry out and have been repeatedly performed with the same results as recorded in figure 3. Of course, the critical temperature for a given species must first be determined. If tests are first carried out at a fairly high temperature at which the strain will die out within a few days and then repeated at gradually reduced temperatures, the organisms will survive for longer and longer periods until finally a point somewhat above the optimum will be reached where the species can be transferred daily for an indefinite period. In figure 3, the nine series on *Escherichia coli* (three at 47° C., three at 46.5°, and three at 46°) certainly would suggest that at about 40° to 42° the species would continue to thrive indefinitely. Furthermore, it suggested to us that critical temperatures might be determined at which a progressive adaptation rather than the death of the species might be observed, provided the organisms were given alternate periods of growth at the optimum temperature. Thus the species would have time to recuperate from each exposure to the higher temperature.

Finally, it should be pointed out that our three test organisms have been kept as stock cultures in the National Institute of Health for years. Never have they failed to grow in ordinary beef-broth media at the optimum temperature (37° C.).

Results With Paramecia

A continuous exposure of a strain of *Paramecium multimicronucleatum* to eosin (10 mg. per milliliter) resulted first in a stimulation of the species, as measured by high population levels, subsequently a gradual weakening, and finally death. This eosin-adapted strain was started on May 18, 1939, and survived for 102 transfers until May 29, 1942, a few days over 3 years. Routine transfers were made every 10 days, or 11 days when the date fell on a Sunday. For the last 20 or more transfers, the organisms were perceptibly smaller and their movements far slower than the controls. At all times the cultures were protected from white light because daylight or ultraviolet will kill paramecia in the presence of eosin in a few hours. Sixteen control series of paramecia cultures, carried along at the same time, have not died.

Another series of cultures of the same organisms have been exposed continuously to methylcholanthrene (1 γ per milliliter) for over 4 years (May 1939 to April 1943). Progress reports on the changed behavior of these methylcholanthrene-exposed organisms have been made (*2, 3*). Briefly stated, the organisms displayed a greater survival time as determined by our survival tests (*2*) after the thirty-fifth transfer. After the fiftieth transfer the population levels were repeatedly higher than control organisms of comparable age. These methylcholanthrene-exposed organisms have now reached the one hundred and fortieth transfer (June 1943); and although they are very small (apparently

one-half size), the population levels continue higher than any of the control series.

Figures 4, 5, and 6 show the daily populations of eosin-adapted and control organisms under identical conditions of partial starvation as described in our survival technique (*2, 3*).

On the fifty-seventh transfer (fig. 4), the eosin-exposed organisms lived longer and reached higher population levels than did the controls. On the eighty-sixth transfer (fig. 5), the eosin-exposed organisms failed to show any increase in population and died on the seventh day. On the ninety-sixth transfer (fig. 6), the organisms decreased rapidly and died on the fourth day while the controls multiplied and survived for more than 15 days. This eosin-exposed strain could not be kept alive after the one hundred and first serial transfer even under optimum conditions.

While the eosin-exposed organisms were finally destroyed in 3 years the methylcholanthrene-exposed are still alive after 4 years. The use of excessive amounts of methylcholanthrene or supersaturated solutions in which the crystals of the hydrocarbon were visible in the culture media have, however, not destroyed either paramecia or bacteria, as far as tests were carried.

The same concentration of phenanthrene (1 γ per milliliter) destroyed paramecia in less than 10 transfers.

A fourth series of paramecia cultures was exposed continuously to crystal violet (1 part in 4,000,000 or 0.4 γ per cubic centimeter). This test was started December 11, 1941. When the twenty-seventh transfer was 25 days old (October 5, 1942), a survival test revealed that the dye-exposed organisms multiplied readily and reached a higher population level than the controls but did not survive for so long a period. From the thirty-first to the thirty-eighth transfer the cultures were becoming progressively weaker, and difficulty was experienced in keeping them alive. Frequently transfers had to be made several times before the new culture would multiply. The thirty-ninth transfer did not survive after repeated transfers from the thirty-eighth (January 14, 1943).

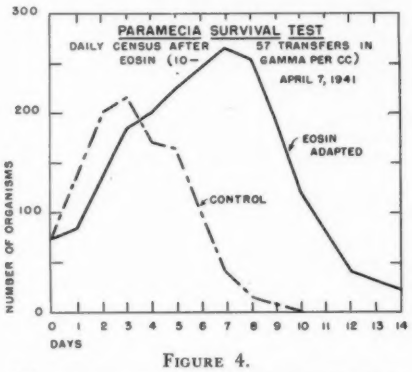

FIGURE 4.

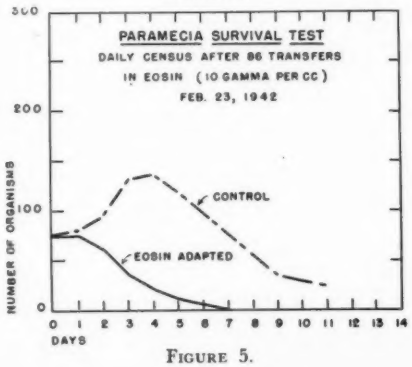

FIGURE 5.

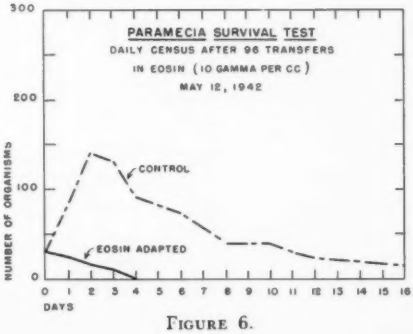

FIGURE 6.

Results With Worms

A culture of a flat worm, *Stenostoma tenuicaudatum*, received from Prof. William A. Kepner, Miller School of Biology, University of Virginia, and identified by Dr. J. W. Nuttycombe, of the University of Georgia, was cultivated in 1,500-ml. covered culture dishes, 1,000 cc. of media to each dish. Each culture was transferred every 14 days to a fresh dish, 12 organisms being used for each transfer. The populations of all dishes were recorded at the end of 45 days. Beginning with the sixth transfer, the amount of culture media was reduced to 500 cc. so that the total population counts would not run excessively high.

The medium is prepared by using 1 ml. of our basic stock paramecia media[1] to 1,000 ml. of distilled water. Twelve grains of wheat are added to each 1,000 cc. and brought to a boil for 1 minute. The freshly prepared medium is then poured into culture dishes and permitted to cool before the dish is inoculated with 2 cc. of media (without worms) from an old culture. The worms are transferred the following day. This permits the bacteria and small rotifers from the old culture to develop. The worms usually find sufficient food in the culture media, but the presence of rotifers seems to enhance the growth rate.

[1] *NaCl, 10.0 gm.; CaCl, 0.6 gm.; KCl, 0.4 gm.; H_2O (distilled), 100.0 ml.*

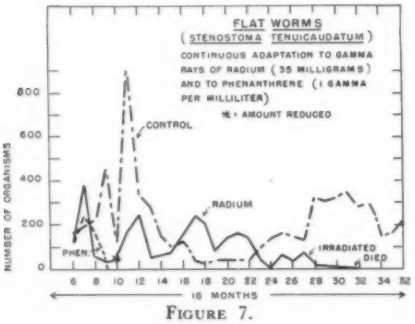

Figure 7.

In figure 7 the organisms were transferred every 14 days over a period of 16 months. The population census was taken regularly 45 days after inoculation of each culture dish.

We have recorded the populations of the controls, the phenanthrene-exposed and the radium-exposed series (35 mg. of radium in 7 platinum-iridium needles). These needles were evenly distributed in small wire cradles on the bottom of the dishes. The phenanthrene was added in a solution of benzene which was spread over the bottom of the dishes and permitted to dry thoroughly before the medium was added. Populations before the sixth transfer were not recorded because the first 5 dishes of each series contained 1,000 cc. and would not have been comparable with subsequent counts in the 500-cc. dishes. It will be noted that the phenanthrene-adapted series died on the ninth transfer. The organisms in the first 8 transfers, extending over a period of 112 days, were apparently healthy and had increased in each dish reaching a population level of 230 organisms on the seventh transfer, which was higher than the corresponding transfer in the control series.

The radium-exposed worms also showed considerable increase in population on the seventh transfer but dropped to as low as 13 organisms on the ninth transfer, barely enough to inoculate the next dish in the series. On the tenth transfer 10 gm. of radium (2 needles) was removed, and thereafter the cultures were for a time more vigorous. However, the series died out on the thirty-second transfer.

From the seventeenth to the twenty-second transfer the populations of the control series were also very low but always contained enough organisms to transfer at the end of each 14-day period. It is believed these low counts were ascribable to the absence of rotifers at that time. We

have no explanation for the extremely high count of the control on the eleventh transfer.

Although the population counts were at all times extremely variable, the tests are certainly suggestive that this species when continuously exposed to unfavorable agents seems to have been little affected at first and may even have been stimulated to some extent, but finally the species was not able to survive.

A methylcholanthrene-exposed series (1 γ per milliliter) of worms which was carried along at the same time but not recorded in figure 7 yielded very high population levels, remaining higher than the controls at all times from the ninth to the twenty-ninth transfer. Both the controls and the methylcholanthrene-exposed worms were alive and vigorous on the fifty-seventh transfer, more than 2 years after the tests were begun.

DISCUSSION

It should be remembered that bacterial species, as a rule, are maintained by simple cell division without any evidence of a sexual cycle. Although some observers have described sexual cycles in these forms, it has not been generally confirmed or accepted. On the other hand, conjugation is frequently seen among paramecia strains. It was observed by us frequently in both our control strains and those exposed to the various agents mentioned. As the treated strains became weaker from continuous exposure, fewer and fewer conjugants were observed and seemed to have disappeared entirely before the species died out. No manifestation of a sexual cycle was ever observed in our strains of worms. Regardless of the presence or absence of sexual reproduction, the various species employed behaved essentially alike when exposed continuously to unfavorable environments over long periods of time. The failure of the carcinogen, methylcholanthrene, to destroy either bacteria, paramecia, or worms, in no way invalidates the principle of continuous exposure. Phenanthrene, which is a noncarcinogenic agent and equally as insoluble as methylcholanthrene, destroyed both worms and paramecia in less than 10 transfers. At present, it is not possible for us to know, prior to testing, whether or not a chemical substance in a given concentration can be considered as a favorable or an unfavorable environment upon a given species.

It should also be remembered that it is difficult to differentiate an individual per se from an individual as an integral part of the species. Nevertheless, our data seem to suggest that the dose of various chemical agents (phenanthrene, eosin, crystal violet) and of various physical agents (temperatures above the optimum, gamma rays of radium) required to kill an individual of a species is greater than that required to destroy the species, provided the exposure is continuous over a sufficient number of generations. The data also suggest a cumulative effect upon the succeeding generations of these organisms.

SUMMARY

Studies upon three species of bacteria (*Eberthella typhosa, Escherichia coli,* and *Streptococcus hemolyticus*), a species of paramecia (*Paramecium multimicronucleatum*), and a species of flat worms (*Stenostoma tenuicaudatum*) seem to support the principle of continuous exposure as defined.

It seemed now obvious to us that discontinuous exposure of species to unfavorable environments as compared with continuous exposure might prove interesting.

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Principles of Species Adjustment
II. Discontinuous Exposure

By R. R. SPENCER, *chief*, M. B. MELROY, *assistant bacteriologist*, and DOROTHY CALNAN, *junior bacteriologist, National Cancer Institute, National Institute of Health, United States Public Health Service*

Since the continuous exposure of three species of actively multiplying bacteria to high temperatures and the continuous exposure of paramecia and worms to certain chemicals had invariably resulted in the death of all individuals of these species after a considerable number of generations, it seemed reasonable to assume that an alternating or discontinuous exposure of a species to an unusual environment might result in a gradual adaptation. The data here recorded support this hypothesis

For the present it was thought best to concentrate our efforts upon bacteria and to limit the type of unfavorable environment to changes in temperature rather than by exposure to chemicals. The reasons for this decision were: (1) The rapidity of cell division of most bacteria under optimum conditions; (2) temperatures can be held constant easily and accurately by thermostatically controlled incubators; (3) chemical environments are not easily controlled or held in constant concentration; (4) chemicals may deteriorate, be absorbed by or broken down by the living cells; (5) when transfers of organisms are made, it is practically impossible to carry over an inoculum from one culture vial to the next in series without transferring at the same time traces of the chemical constituents; such technique could not be called a strictly alternating exposure; and (6) fluctuations in temperature are perhaps the most common and universal environmental changes in nature to which living things are subjected.

The materials and methods here employed are, in general, identical with those described in the first paper of this series (*1*). The results charted in the two figures 1 and 2 warrant, we believe, the posing of our second principle:

By discontinuous or alternating exposure a species can successfully adapt to the same and by degrees to a greater intensity of the same environment that is fatal when the exposure is continuous.

RESULTS

In figure 1, A, are charted the daily transfers of *Escherichia coli* beginning with the twenty-seventh day, at which time the strain was incubated first at 45° C. for 24 hours. The first 26 serial daily transfers had been incubated at 37° for 24 hours, and just before transferring to the next vial each culture had been completely immersed in boiling water for 10 seconds. Beginning with the twenty-seventh transfer, the following odd-numbered cultures were incubated at 45° and the even-numbered at 37° for 24 hours each. The sixty-first culture was the first to be incubated at 47.5°; the one hundred and seventy-fifth was the first to be incubated at 49°; and the three hundred and eighty-sixth was the first to be incubated at 50°. This series is still being alternately incubated daily between 37° and 50° and has now reached the four hundred and sixtieth daily transfer. It is still a gram-negative, motile bacillus which agglutinates in *E. coli* immune serum in a dilution of 1 : 640.

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In figure 1, B, are charted 14 *Escherichia coli* series that were transferred daily and exposed continuously to a temperature of 47° C. All of the first 10 controls died in 3 days, a result identical with previous tests upon normal *E. coli* when exposed continuously to this temperature. In contrast the last 4 series (Nos. 11, 12, 13, and 14) were inoculated from the four hundred and twentieth vial of the heat-adapted series recorded under A. After surviving 50 days of continuous exposure to 47°, the organisms in these four series were proven to be pure cultures of *E. coli* by specific agglutination. Whether or not these heat-adapted organisms would have lived indefinitely at 47° is, of course, not known. They had not perceptibly weakened during the 50 transfers, as measured by the degree of turbidity, but the growth was never so heavy as that of normal cultures.

Inasmuch as the inoculum employed was always the same (0.5 ml.) and all of the first 10 control series were planted from a 24-hour broth culture of *Escherichia coli,* a total of 5 cc. or at least 5 billion organisms were planted, and none of them or their progeny were able to survive beyond the third transfer at 47° C. It would seem that this excludes the possibility that the survival of the last 4 heat-adapted series of *E. coli* for 50 days at the same temperature was due to a process of selection of 1 or more organisms and their progeny from the A series that were capable from the beginning of withstanding a temperature of 50° for 24 hours. In further support of this contention, it should be pointed out that when the A series was growing well at 47.5°, repeated attempts failed to obtain growth at 49°. It was not until the one hundred and seventy-fifth transfer that the strain would survive for 24 hours at 49°.

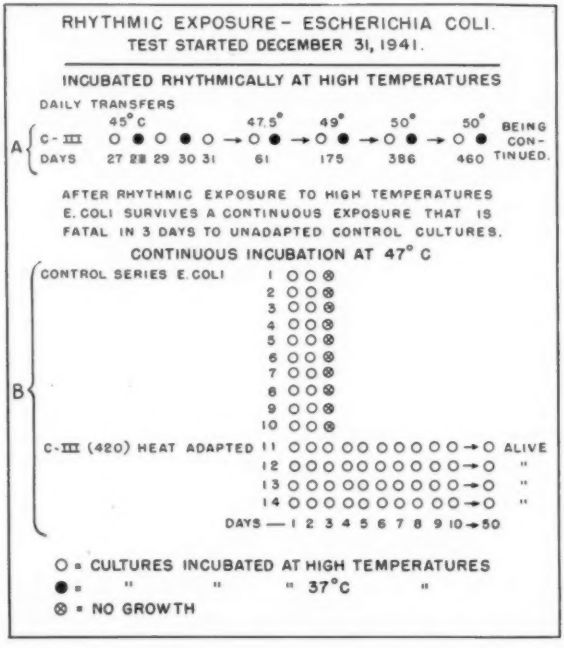
RHYTHMIC EXPOSURE – ESCHERICHIA COLI.
TEST STARTED DECEMBER 31, 1941.
INCUBATED RHYTHMICALLY AT HIGH TEMPERATURES
DAILY TRANSFERS
45° C
47.5°
49°
50°
50°
A
C-III
DAYS 27 28 29 30 31 61 175 386 460
BEING CONTINUED.
AFTER RHYTHMIC EXPOSURE TO HIGH TEMPERATURES
E. COLI SURVIVES A CONTINUOUS EXPOSURE THAT IS
FATAL IN 3 DAYS TO UNADAPTED CONTROL CULTURES.
CONTINUOUS INCUBATION AT 47° C
B
CONTROL SERIES E. COLI
C-III (420) HEAT ADAPTED
DAYS — 1 2 3 4 5 6 7 8 9 10 → 50
ALIVE
O = CULTURES INCUBATED AT HIGH TEMPERATURES
● = " " " 37°C "
⊗ = NO GROWTH

FIGURE 1.

Likewise, when successful growth was obtained at 49°, many subsequent attempts to grow the strain at 50° ended in failure. Successful growth and viable organisms, withstanding 50° for 24 hours, were not obtained until the three hundred and eighty-sixth daily transfer, more than a year after the discontinuous heat-exposure process was started. The results, therefore, suggest a gradually and cumulatively acquired ability over thousands of cell-division cycles to endure and thrive at a temperature of 50°. Unquestionably, however, potentially heat adaptable cells and their progeny did undergo a gradual process of selection.

In figure 2 are charted the results of four additional series of transfers employing another species, *Eberthella typhosa.* All four series, A, B, C, and D, were started from the same 24-hour broth cultures on the same day. The amount of inoculum transferred was always 0.5 ml., and sterile tuberculin syringes were used. The first series, A, served as a control and was incubated continuously at 45° C. As expected with continuous treatment, the organisms finally failed to grow. There was no growth in the forty-sixth vial.

In series B, C, and D, however, the organisms were rhythmically exposed to high temperatures starting at 45°. It will be noted that series B incubated every other day at a high temperature was still alive and thriving at the four hundred and thirtieth transfer, at which time it was being exposed to 50° on alternate days. However, the even-numbered vials incubated at 37° have grown more vigorously and shown greater turbidity than the odd-numbered vials incubated at 50°, and the organisms in the four hundred and thirtieth transfer of the B series were still gram-negative motile rods which agglutinate in specific *Eberthella typhosa* immune serum in a dilution of 1: 1280.

Series C and D were incubated at high temperatures every 2 and 3 days, respectively. Series C died on the seventy-sixth transfer or the one hundred and fifty-sec-

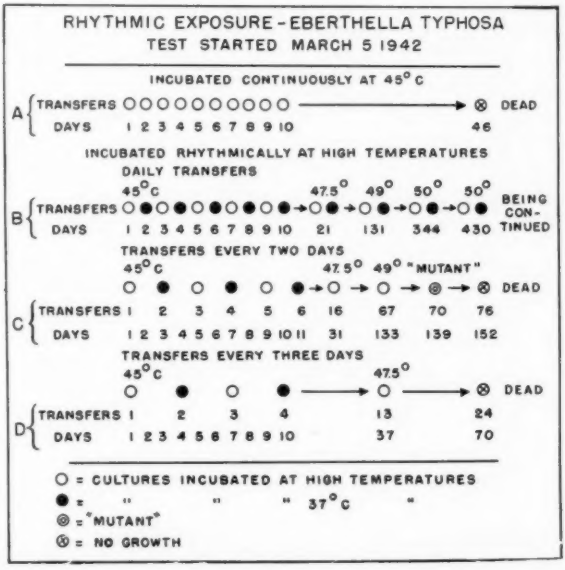
RHYTHMIC EXPOSURE - EBERTHELLA TYPHOSA
TEST STARTED MARCH 5 1942
INCUBATED CONTINUOUSLY AT 45° C
A
TRANSFERS ○○○○○○○○○○ → ⊗ DEAD
DAYS 1 2 3 4 5 6 7 8 9 10 46
INCUBATED RHYTHMICALLY AT HIGH TEMPERATURES
DAILY TRANSFERS
45°C 47.5° 49° 50° 50°
B
TRANSFERS ○●○●○●○●○●→○●→○●→○●→○● BEING CONTINUED
DAYS 1 2 3 4 5 6 7 8 9 10 21 131 344 430
TRANSFERS EVERY TWO DAYS
45°C 47.5° 49° "MUTANT"
○ ● ○ ● ○ ●→○ → ○ → ◎ → ⊗ DEAD
C
TRANSFERS 1 2 3 4 5 6 16 67 70 76
DAYS 1 2 3 4 5 6 7 8 9 10 11 31 133 139 152
TRANSFERS EVERY THREE DAYS
45°C 47.5°
○ ● ○ ● → ○ → ⊗ DEAD
D
TRANSFERS 1 2 3 4 13 24
DAYS 1 2 3 4 5 6 7 8 9 10 37 70
○ = CULTURES INCUBATED AT HIGH TEMPERATURES
● = " " " 37°C "
◎ = "MUTANT"
⊗ = NO GROWTH

FIGURE 2.

ond day. Series D died on the twenty-fourth transfer, or the seventieth day. The survival of the B series and the death of series C and D demonstrate, as one would reasonably expect, the importance of the duration of the exposure to and the interval of rest or freedom from high temperatures. However, in contrast with the A series, there may be seen definite evidence of adjustment in each of the three rhythmically exposed series.

In the C series the next morning after inoculation, the seventieth transfer was found with the rubber stopper blown out because of the formation of gas. Gram-positive as well as gram-negative organisms were observed in the smears.

This series was continued, however, by going back and planting again from the sixty-ninth vial. The stoppers were now prevented from blowing out by removing a considerable amount of the air with a sterile hypodermic syringe. Nevertheless, the growth failed to appear six transfers (12 days) later in vial 76. Upon analysis the gas formed proved to be CO_2. The stock broth medium used was sugar-free except perhaps for small traces of muscle sugar. Up to and including vial 69, stained smears had been made nearly every day at the time transfers were made and when each culture was only 24 hours old. Weeks later, when the cultures in this series had aged considerably, gram-positive forms could be demonstrated in many vials from the twenty-fifth to the seventieth, but not in all. This gram-positive form could not, however, be isolated from the normal typhoid organisms in the cultures. When plated out repeatedly only one type of typical typhoid colony was observed, but smears would show here and there a gram-positive form. Finally it was found that by heating such mixed cultures to 56° for 1 hour, transfers would then invariably fail to grow on agar slants; only an occasional transfer would grow in plain broth, and a great majority of transfers to broth covered with oil (anaerobic) would yield a good growth. Examination of the smears of such broth cultures still showed mixed gram-positive and gram-negative forms. This strain, which had been separated from the normal typhoid organisms by heating to 56° for 1 hour, has never grown on agar surfaces either under aerobic or anaerobic conditions. Such behavior is unlike air-contaminating organisms. Furthermore, the broth cultures of this strain die out when left for a few weeks at room temperature. It is not agglutinated by specific *Eberthella typhosa* antiserum, is nonmotile, and ferments the following sugars with acid and gas formation: dextrine, dextrose, galactose, glycerol, inisitol, inulin, lactose, levulose, maltose, mannose, raffinose, starch, sucrose, and trehalose. Indeed, gas is frequently formed in sugar-free broth; and although growth will not occur when planted heavily on agar surface, the inoculum seeps down between the glass tube and the agar where some growth does occur, and the sugar-free agar is split into numerous parts by gas formation. Another striking characteristic of this organism, as compared with the normal *E. typhosa* from which it apparently arose, is the rapidity of growth and the accelerated rate of cell division in broth. Within 2 or 3 hours there is marked turbidity as compared with control typhoid cultures. Its behavior and general characteristics do not fit that of any organism classified in Bergey's Manual of Determinative Bacteriology. We have tentatively labeled this organism a "mutant" for the lack of a better term. It seems to be a permanently altered form which has not reverted to the original typhoid strain. It is recognized that the term "mutant" implies, to the geneticist at least, a chromosomal change in an organism that undergoes a sexual cycle. Sexual cycles and chromo-

somal structures have not been convincingly demonstrated for bacteria.

By following closely the same technique and employing the same 2-day-rhythm exposure (fig. 2, C), we have failed to obtain this or similar mutants from *Eberthella typhosa*. In one instance the organisms died on the sixteenth transfer; and in the other, on the eighteenth transfer (36 days).

DISCUSSION

It is believed that our data in this and the preceding paper (*1*) upon three species of bacteria represent the first controlled experimental results which have contrasted the striking difference in the end results of a continuous as compared with a discontinuous exposure of actively multiplying species to unusual environments of the same intensity. On the one hand, the result is fatal to the species but not immediately fatal to individuals of the species. On the other hand, the species is able to adjust successfully to the new environment and by degrees to higher intensities of the same environment. Whether or not these principles can be experimentally demonstrated to hold for higher forms we do not know. It seems reasonable to assume that they do, for fluctuation or rhythmicity of environmental factors is a universal experience of all living things. Periods of rest, recuperation, or regeneration seem to be essential to survival in the process of adaptation for either the individual or the species. On the other hand, continuous exposure to an unfavorable or unusual environment of even low intensity precludes a period of rest or freedom from a specific environmental factor and thus may be fatal. Very likely a species exposed even continuously to unfavorable environmental conditions of extremely low intensity or to toxic substances in extremely dilute amounts can adapt to such conditions. Nevertheless, our tests indicate that certain conditions which are apparently harmless throughout the normal life span of an individual may be fatal finally to the species by means of cumulative effects. What these apparently gradual and steplike changes are remains to be investigated.

In this connection it is recalled that Magoon (*2*), of the United States Department of Agriculture, Bureau of Plant Industry, showed that, contrary to the commonly accepted idea, bacterial spores once formed have a fixed resistance, have in reality a resistance which varies with age and with the conditions under which the spores exist. He was able to demonstrate that bacterial spores of *Bacillus mycoides* derived from heat resistant survivors in thermal-death-time tests possessed higher resistance to heat than did the original spores, and by a process of selection a strain of bacteria was obtained of which the spores attained a resistance to heat at least 25 times that of the original spores. Magoon was interested in determining the heat necessary to destroy spore-bearing organisms in canned goods.

Armstrong (*3*), of the United States Public Health Service, attempted to develop a vaccine virus that would keep at room or body temperatures. He exposed the virus to incubator temperatures (37.5° C.) and by continued selection and propagation of the virus in the rabbit was able to develop a strain which showed an "increase of several hundred percent in the period of time during which it will withstand a temperature of 37.5° C. and still give typical skin takes on rabbits." A notable increase in the virulence of the virus for animals was also noted, and this feature deterred him from employing the strain in man.

Neither Magoon nor Armstrong mentioned the principle of rhythmic exposure

to an unfavorable environment as a possible factor of broad application in the study of the mechanism of species adaptation, although their technique involved this principle.

Huntington (*4*) is the only investigator of whom we are aware, who has presented data and observations emphasizing the importance of an alternating environment upon a species. Huntington's thesis is that the highest human civilizations have arisen only in areas where there is an alternating climate. Continuously hot or continuously cold climates are enervating; a changeable climate is stimulating. A thorough search of the biologic literature may reveal other examples that, in the light of our experiments, may now suggest the general biologic importance of rhythmic exposure to unfavorable environments.

Hegarty and Randall (*5*) found that by serially transferring *Escherichia coli* in nutrient broth every 4 hours (thus keeping the organisms in the logarithmic phase of growth and obliterating the lag phase) the strain died out after the forty-second transfer. It was concluded that the "lag phase was a rejuvenation process as originally suggested by Sherman and that it is necesary for the continued growth of a bacterial culture." In the light of our experience, it would seem equally reasonable to interpret the dying out of the species as being due to the fact that the bacterial cells were never permitted to age or mature but were kept continuously in an actively multiplying phase. The conditions of the experiment placed the burden of carrying on the species upon young, immature cells only. Respite from active multiplication and maturity seem to us to be more likely as "necessary to the continued growth of a bacterial culture" than the lag phase.

Finally, we suggest that the genesis of cancer in the organized tissue cells of mammals may involve a mechanism not unlike that we have here demonstrated in the case of free-living, single-cell forms.

In fact, Cramer and Stowell (*6*), in a recent publication appearing since our tests were completed, have shown that skin cancer can be induced by much smaller doses if the applications of methylcholanthrene are made at long intervals, such as 1 month. The continuous exposure of the skin of the mouse, which involves relatively large total doses in order to induce skin cancer, is not essential.

Investigators in the field of cancer have always insisted that chronic irritation alone is not sufficient to explain the genesis of cancer since some of the most irritating chemicals have not been shown to be carcinogenic and some of the least irritating are known to be highly carcinogenic. It is our belief that by properly utilizing the discontinuous method of exposure many substances which cause sublethal injury may be found to be carcinogenic, although heretofore they have not even been suspected of having carcinogenic properties. Experiments now being carried on at this laboratory by Dr. Eschenbrenner [1] will reveal the application of the discontinuous principle in testing the carcinogenicity of toxic substances of simple but diverse chemical structure.

SUMMARY

Data are presented on two species of bacteria, *Eberthella typhosa* and *Escherichia coli,* in support of the principle that discontinuous exposure of an organism to an unfavorable environment (high temperatures) results in a gradual adaptation of the species. Whether or not such adaptations will regularly or merely occasionally result in permanent changes in

[1] *To be published in the next issue of this Journal.*

structure and function is not known at present. At least one such change is here recorded.

A possible connection between the adaptive process in bacteria and the carcinogenic process in mammals is suggested.

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Experimental Chemotherapy of Tumors in Mice

By Floyd C. Turner, *senior surgeon, National Cancer Institute, National Institute of Health, United States Public Health Service*

Experiments on the chemotherapy of cancer are, perforce, largely empirical since facts concerning the causes, biology, and biochemistry of neoplastic growth are few and confusing. A large number of chemicals has been tested for tumor-inhibiting qualities with discouraging results (*1–11*). Despite the absence of logical approach, the successes of chemotherapy in other fields stimulated the undertaking of a program on the chemotherapy of tumors in mice. Since 1936 a total of 977 substances and mixtures of substances has been tested in 18,395 tumor-bearing mice. No effective remedy has been found, but it is felt that a summary of part of these investigations is appropriate since the work has been temporarily suspended.

MATERIALS AND METHODS

Investigations in the chemotherapy of cancer involve several factors: (1) The materials to be tested; (2) the test animal and the test neoplasm; and (3) the method of administering the test material.

Test Materials

The present status of our knowledge of the neoplastic process necessitates an empirical selection of the chemicals to be tested. Essentially, the choices are based on the hope that a compound may be encountered that will selectively injure and ultimately destroy neoplastic tissues and yet be relatively harmless to the normal cells of the body.

The substances tested were usually chosen with the hope of affecting some specific system in the neoplasm. For example, a series of phenanthrene derivatives were tested (*12*) because they are related to colchicine, which affects mitoses, and because mitoses are more frequent in tumors than in normal adult tissues. Other series of compounds were tried because they were known to inhibit certain enzyme systems or enzymes that depend on –SH groups for activity. Since the results were negative, a report on pre-experimental attempts to give a rational basis for the use of the compounds seems unnecessary.

The test materials were dissolved or suspended in distilled water or physiologic saline solution, in such concentrations that 0.5 cc. or less was the total volume injected. Some of the long-chain hydrocarbons were liquefied by gentle heat and dispersed by the use of Metamucil, which contains a mucilloid from psyllium seed and a dextrose. The majority of the naphthalene, anthracene, and benzanthracene derivatives were dissolved in filtered lard.

Test Animals and Tumors

The availability and the ease of maintenance make the mouse a desirable animal to use in experiments that require large numbers of animals. Also, in the mouse there is a wide variety of spontaneous, induced, and transplanted tumors from which selection can be made.

Mice with cancer of spontaneous origin appear to be most desirable for therapeutic experiments since under such conditions

the neoplasm arises from the host tissues and is subjected to the normal influences of the body metabolism. Mice with spontaneous tumors were used when available; in all, 2,568 such animals were used in 351 tests. Nearly all of the tumors were found to be mammary adenocarcinomas. They occurred in mice of strains C3H, A, Swiss, and dba, and in hybrid mice. Rates of growth and of spontaneous regression were determined in 2 strains. No spontaneous regressions were observed in 46 C3H mice or in 56 Swiss mice; however, other experiences indicate that a small percentage of subcutaneous tumors in Swiss mice undergoes spontaneous regression.

The great majority of these experiments were performed on mice bearing transplanted tumors because they can be produced quickly in any desired number. Sarcoma 37 in strain A backcross mice, implanted directly into the skin of the abdomen according to a technique previously described (*12*), was used to the greatest extent. The regression rate of sarcoma 37 in these mice was found to be 12 percent in 220 animals. Seven other tumors, transplanted subcutaneously into mice of strains in which the tumors originated, and sarcoma CR 180 in strains A, C, C3H, and dba mice were also used: mammary adenocarcinoma and glioma in strain C3H mice, pulmonary adenocarcinoma, intestinal adenocarcinoma and leukosis 72942 in strain A mice, salivary squamous-cell carcinoma in strain C mice, and leukosis Y 103 in strain dba, subline 212, mice. None of these tumors regressed spontaneously. Mice of both sexes were used at random and were 1 to 3 months old at the time of inoculation. All animals were maintained on Purina dog chow pellets and had access to an unlimited supply of water. In all, 15,827 mice with transplanted tumors were used in 1,250 tests, or an average of 12 mice per test.

Test Method

The usual method of testing the therapeutic effect of compounds upon experimental tumors was by the injection of the substance at a site distant from the tumor. Since general therapeutic effects were desired, it is obvious that the injection of material into the tumor itself would not fulfill the requirements of the test.

The injections were usually intraperitoneal, although in some tests the subcutaneous, intravenous, oral, and inhalation routes also were used. The effect of a single injection (of various concentrations) was usually tried first to determine tolerance. Subsequently, the injections were given daily (except Sundays) and were continued until the death or impending death of the tumor-bearing mice. The dosages used were as large as the mice would tolerate, usually about 50 percent of the minimal lethal dose, although the doses were adjusted from time to time to the tolerance of the animals. The size of the dose was occasionally limited by the amount of available material.

RESULTS

Table 1 summarizes the results obtained with 132 compounds that were given repeatedly to mice bearing spontaneous mammary carcinomas. It is impractical to present the results on the transplanted tumors and the effects obtained with single injections of other compounds since on the whole the results were negative. The desired result was the total and permanent regression of the tumor in a healthy animal, but other effects were also recorded. The slowing of the rate of growth of the tumors was considered significant only if the animals remained in good condition.

TABLE 1.—*Experimental treatment of spontaneous mammary tumors in mice*

Chemical	Strain of mouse	Number of mice	Dose per injection	Number of injections	Route [1]
			Milligrams		
Inorganic compounds:					
Copper oxide	C3H	6	1.0	53	IV.
Copper sulfate	C3H, Swiss	5	.05	84	IP.
Do	C3H, Swiss	8	.05	46	IV.
Iron, colloidal	C3H	5	2.5	49	IP.
Do	C3H	4	.05	19	IV.
Sodium chloride	C3H, Swiss	10	50	18	IP.
Sodium selenite	C3H, Swiss	5	.05	7	IP.
Do	C3H	5	.01	42	IP.
Water, distilled	A, Swiss	17	200	32	IV.
Organic compounds:					
Aliphatic:					
Acetone	C3H, Swiss	4	400	24	Inhaled.
Aerosol O. T. (dioctyl sodium sulfosuccinate)	C3H	10	.1	24	IP.
n-Arachidic acid	C3H, Swiss	8	25	2	IP.
Azelaic acid	Swiss	7	10	2	IP.
Calcium pantothenate	C3H, Swiss	6	2	29	IP.
Caprie acid	C3H, Swiss	6	25	2	IP.
Caprylic acid	Swiss	7	2	2	IP.
Cerotic acid	C3H	7	25	2	IP.
Chlormethylmercury chloride	C3H	4	.12	40	IP.
Do	C3H, Swiss	12	.005	17	IV.
Diselenobisacetic acid	C3H, Swiss	8	.2	46	IP.
Erucie acid	C3H, Swiss	6	.8	8	IP.
Formaldehyde gas	Swiss	3	.27	11	Inhaled.
Glucose	C3H	7	20	37	IP.
d-Glutamic acid	C3H	9	10	53	SC.
Glycine	C3H	4	12	39	IP.
Heptylic acid	C3H, dba	7	5	2	IP.
α-Hydroxylauric acid	C3H, Swiss	8	1	4	IP.
α-Hydroxymyristic acid	C3H, Swiss	[2] 6	25	3	IP.
αHydroxypalmitic acid	C3H	7	5	4	IP.
12-Ketostearic acid	C3H, Swiss	[2] 9	25	3	IP.
Margaric acid	C3H, Swiss	8	25	3	IP.
Mesityl oxide	Swiss	2	[3] .07	7	Inhaled.
Methylhexylketone	A	3	[3] .7	36	Do.
Methyl-*n*-dodecylacetic acid	C3H, dba	8	25	2	IP.
Myristic acid	C3H, Swiss	7	25	3	IP.
n-Nonadecanoic acid	C3H, Swiss	[2] 8	25	2	IP.
Nonylaldehyde	C3H, Swiss	3	[3] .5	8	Inhaled.
Octylaldehyde	C3H, Swiss	2	[3] .48	10	Do.
Palmitic acid	C3H	7	25	3	IP.
Pelargonic acid	Swiss	7	10	2	IP.
n-Pentadecanoic acid	do	8	25	2	IP.
n-Propyl-*n*-tetradecylacetic acid	C3H, Swiss	8	24	3	IP.
Putrescine	C3H	7	10	33	SC.
Sebacic acid	C3H	6	5	7	IP.
Stearic acid	C3H, Swiss	16	7.7	5	SC.
Tetrabromostearic acid	C3H	7	24	4	IP.
Tridecylic acid	A, dba	7	10	3	IP.
Triethanolamine	C3H	5	10	56	SC.
Triethanolamine hydrochloride	C3H	4	10	55	IP.
Undecyl aldehyde	Swiss	2	[3] .5	19	Inhaled.
Undecylic acid	A, C3H	8	10	2	IP.
Urea	C3H, Swiss	7	50	21	SC.
Simple benzene, naphthalene, and anthracene derivatives:					
4-Amino-2-methyl-1-naphthol	C3H, Swiss	8	.25	18	IP.
4-Amino-2-methyl-1-naphthol hydrochloride	C3H, Swiss	8	.5	21	IP.
1-Amino-2-naphthol-4-sulfonic acid	C3H, Swiss	8	2.5	34	IP.
p-Chloromercuribenzoic acid	C3H	8	1	60	IP.
Diformaldehydesulfoxylate diaminoph enylsulfone	C3H	5	5	57	IP.
Di-α-naphthyldianisylethane	C3H, Swiss	8	.5	61	IP.
Insa (monoethanolamine salt of 7-iodo-1-hydroxy-naphthalene-3, 6-disulfonic acid.	C3H	8	20	40	IP.
o-Mercuribenzoic anhydride	C3H, Swiss	7	.5	73	IP.
Metaphen (4-nitroanhydro-hydroxymercuri-o-cresol).	Swiss	8	.02	21	IP.
p-Methylaminophenol sulfate	C3H	7	.25	17	IP.
9-Methylanthracene	C3H	6	2	42	IP.
Neoprontosil	C3H, Swiss	6	10	51	IP.
4-Nitro-4′-acetylaminodiphenylarsinic acid	Swiss	10	1	25	IP.
4-Nitro-4′ acetylaminodiphenylarsinoxide	C3H	5	.05	15	IP.
4-Nitro-4′-aminodiphenylarsinic acid	Swiss	5	.5	23	IP.
4-Nitro-4′-aminodiphenylsulfide	C3H	5	5	11	IP.
4-Nitrophenylarsonic acid	C3H, Swiss	6	.1	12	IP.
Trypan blue	C3H	20	2.5	13	IP.

See footnotes at end of table.

TABLE 1.—*Experimental treatment of spontaneous mammary tumors in mice*—Continued

Chemical	Strain of mouse	Number of mice	Dose per injection	Number of injections	Route [1]
Organic compounds—Continued.					
Phenanthrene derivatives:			*Milligrams*		
2-Acetoxy-7-acetyl-9,10-dihydrophenanthrene	C3H	9	0.5	18	IP.
3-Acetoxyphenanthrene-4-aldehyde	C3H, Swiss	11	.5	39	IP.
9-Acetyl-s-octahydrophenanthrene	C3H, Swiss	8	.66	51	IP.
9-(2-amino-1-hydroxyethyl)-3-octahydrophenanthrene hydrochloride.	C3H	11	.5	44	IP.
3-(2-amino-1-hydroxy-*n*-propyl)-phenanthrene hydrochloride.	C3H	8	.5	52	IP.
9-Aminomethyl phenanthrene hydrochloride	C3H	14	.1	78	IP.
9-(2-amino-1-oxopropyl)-s-octahydrophenanthrene hydrochloride.	C3H, Swiss	8	.5	54	IP.
2-(2-amino-1-oxopropyl)-phenanthrene hydrochloride.	C3H	8	.5	43	IP.
3-(2-amino-1-oxopropyl)-phenanthrene hydrochloride.	C3H	12	.5	36	IP.
3-(2-bromo-1-oxopropyl)-phenanthrene	C3H	8	.5	44	IP.
3-(2-diethylamino-1-oxoethyl)-phenanthrene	C3H	6	.5	23	IP.
3-(2-diethylamino-1-oxoethyl)-phenanthrene hydrochloride.	C3H	6	.41	75	IP.
3-(2-dimethylamino-1-oxopropyl)-phenanthrene-hydrochloride.	C3H	6	.5	48	IP.
9-(2-dimethylamino-1-oxopropyl)-phenanthrene hydrochloride.	C3H	13	.5	53	IP.
3-(2-ethylamino-1-oxopropyl)-phenanthrene hydrochloride.	C3H, Swiss	8	.01	44	IP.
9-Ethylphenanthrene	C3H	7	.5	34	IP.
2-Hydroxy-7-acetyl-9,10-dihydrophenanthrene	C3H	8	.5	27	IP.
3-Hydroxy-6-acetylphenanthrene	C3H, Swiss	6	.5	36	IP.
2-(1-hydroxyethyl)-phenanthrene	C3H	8	.5	65	IP.
3-(1-hydroxyethyl)-phenanthrene	C3H	7	.5	36	IP.
9-(1-hydroxyethyl)-phenanthrene	C3H	5	.5	46	IP.
2-Hydroxyphenanthrene-1-aldehyde	C3H	7	.5	40	IP.
9-Hydroxyphenanthrene-10-aldehyde	C3H, Swiss	6	.5	5	IP.
2-Hydroxy-7-propionyl-9,10-dihydrophenanthrene	C3H	8	.5	37	IP.
3-(2-isonitroso-1-oxopropyl)-phenanthrene	C3H	8	.5	27	IP.
3-Methoxy-6-acetylphenanthrene	C3H, Swiss	12	.5	36	IP.
3-Methoxy-9-(2-dimethyamino-1-oxoethyl)-phenanthrene hydrochloride.	C3H	6	.5	34	IP.
3-(2-methylamino-1-hydroxy-*n*-propyl)-phenanthrene hydrochloride.	C3H	8	.5	37	IP.
2-(2-methylamino-1-oxopropyl)-phenanthrene hydrochloride.	C3H	8	.5	57	IP.
3-(2-methylamino-1-oxopropyl)-phenanthrene hydrochloride.	C3H, Swiss	8	.5	43	IP.
9-(2-methylamino-1-oxopropyl)-phenanthrene hydrochloride.	Swiss	[2] 6	2.5	74	IP.
Phenanthrene	C3H	8	.5	55	IP.
Phenanthrene-9-carboxylic acid	C3H	14	.5	55	IP.
2-(2-piperidino-1-hydroxyethyl)-phenanthrene hydrochloride.	C3H	8	.5	34	IP.
2-(2-piperidino-1-hydroxy-*n*-propyl) phenanthrene.	C3H	8	.5	68	IP.
2-(2-piperidino-1-oxoethyl)-phenanthrene hydrochloride.	C3H	6	.5	32	IP.
3-(2-piperidino-1-oxoethyl)-phenanthrene hydrochloride.	C3H, Swiss	7	.25	69	IP.
9-(2-piperidino-1-oxoethyl)-phenanthrene hydrochloride.	C3H	11	.5	44	IP.
3-(2-piperidino-1-oxopropyl)-phenanthrene hydrochloride.	C3H	8	.24	43	IP.
9-Propionyl-s-octahydrophenanthrene	C3H	10	.5	61	IP.
2-Propionylphenanthrene	C3H, Swiss	11	.5	46	IP.
3-Propionylphenanthrene	C3H, Swiss	7	.5	81	IP.
9-Propionylphenanthrene	C3H, Swiss	8	.5	42	IP.
Other carbocyclic derivatives:					
Chaulmoogric acid	A, C3H	8	10	3	IP.
Ninhydrin	C3H, Swiss	6	.5	26	IP.
Vitamin D_2	C3H	6	20	38	IP.
Complex polysaccharides, steroids, and proteins:					
Acenaphthene cholcic acid	C3H	7	1	71	IP.
Digitonin	C3H, Swiss	8	.5	23	IP.
Protamine	C3H, Swiss	6	.5	28	IP.
Saponin	C3H	7	.25	33	IP.
Codeine, thebaine, and morphine derivatives:					
des-N-methyldihydrothebainone	C3H	4	5	13	SC.
Desoxymorphine-C hydrochloride	C3H	4	5	13	SC.
Dihydro-des-N-methyldihydrohydroxycodeine-B	C3H	2	5	10	SC.
Dihydro-des-N-methyldihydrothebaine hydrochloride.	C3H	4	5	12	SC.
Dihydro-des-N-methyldihydrothebainone	C3H	4	5	13	SC.

See footnotes at end of table.

TABLE 1.—*Experimental treatment of spontaneous mammary tumors in mice*—Continued

Chemical	Strain of mouse	Number of mice	Dose per injection	Number of injections	Route [1]
Organic compounds—Continued.					
Codeine, thebaine, and morphine derivatives—Con.			*Milligrams*		
Dihydrothebainone hydrochloride	C3H	4	5	14	SC.
Ethyldihydrothebainone hydrochloride	C3H	6	.5	29	IP.
Isomethyl-7-ketodihydrothebainone	C3H	7	.1	47	IP.
Other heterocyclic compounds:					
Guanine	C3H, Swiss	8	5	31	IP.
Guanosine	C3H	7	1	27	IP.
7-Hydroxydibenz- [f, h]-quinoline	C3H	8	.5	32	IP.
Indole	C3H, Swiss	8	1	21	IP.
Porphyrexide	Swiss	[2]6	5	4	IV.
Riboflavin	C3H	9	1	35	SC.
Sulfathiazole	C3H, Swiss	6	10	52	IP.
Thiamin chloride	C3H	7	1	37	IP.

[1] Key to route: IP, intraperitoneally; IV, intravenously; SC, subcutaneously.

[2] 1 tumor in Swiss mouse regressed.

[3] Cubic centimeter.

Regression of mammary carcinoma of spontaneous origin in mice of the Swiss strain is recorded in single instances with five compounds: α-hydroxymyristic acid, 12-ketostearic acid, *n*-nonadecanoic acid, porphyrexide, and 9-(2-methylamino-1-oxopropyl)-phenanthrene hydrochloride. These findings are not considered as definitive, although no spontaneous regressions were encountered in the control animals of the Swiss strain bearing similar tumors. None of the mammary tumors in C3H mice regressed.

Regression of approximately one-fourth of leukosis Y 103 with sodium selenite (0.02 mg. given 9 times intraperitoneally) and of sarcoma CR 180 with lactic acid (5 mg. injected 14 times intraperitoneally) were also in contrast with the lack of spontaneous regressions in their control groups. Retardation in the rate of growth of sarcoma 37, as well as total regression in 20 to 35 percent of strain A backcross mice, was encountered with 9 of 14 derivatives of anthracene and benzanthracene which were dissolved in lard and injected once intraperitoneally.

Despite the essentially negative results, it is felt that further testing of chemicals and combinations of chemicals is well worth while, despite the empirical basis on which such work must proceed until further knowledge of the fundamental aspects of the cancer problem permits a more rational approach.

It is assumed that therapeutic effects attained in a mouse bearing a cancer may be applicable, or at least produce a clue, to the treatment of neoplasms in other species, including man. It has been emphasized by many workers that this assumption is not necessarily warranted. It is probable that cancer is not a single disease but a large and complex group of diseases, and that the effectiveness or ineffectiveness of a compound on a specific type of tumor in a mouse may not be substantiated on other types of tumors or in other species. A practicable experimental method is the use of several types of tumors in testing a given compound, and the definite implication that the results are applicable only under the specific conditions of the experiment.

SUMMARY

Results are presented on the testing of 132 chemical compounds in the experimental chemotherapy of spontaneous mammary carcinoma in mice. No effective remedy was discovered.

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Enzymatic Activity of Normal Adult, Regenerating, Fetal, and Neoplastic Hepatic Tissues of the Rat

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Study of the properties of the hepatomas is facilitated by the ready availability of at least three different kinds of homologous hepatic tissue with which the hepatomas may be compared. These tissues are normal adult, regenerating, and fetal liver. The last two tissues, like the hepatomas, are rapidly growing at rates which in certain stages may even exceed that of the hepatomas, but unlike the hepatomas, this growth rate is controlled.

Extensive comparative studies of the quantitative activity of several enzymes in hepatomas, adult, and regenerating livers revealed that whereas the last two tissues possess, with few exceptions, nearly identical enzymatic activity, the hepatomas, with few exceptions, possess enzyme systems of a considerably different order of magnitude (*1–9*). Comparison of the activity of fetal with adult livers revealed on the whole a relation resembling that between the hepatomas and normal adult or regenerating liver (*1, 3, 5*). Some of the enzyme systems were examined in rat fetal liver (*8, 9*); most of them were examined in rabbit fetal liver (*1, 5*); in all cases the data obtained were compared with those for the normal and neoplastic hepatic tissues of rats and mice. For valid comparisons it would be desirable to possess all such information on the tissues of a single species of animal. For this reason, enzymatic studies made earlier on rabbit fetal tissues were repeated on the fetal livers of rats; in addition, new enzyme systems were studied. Together with the data adduced earlier on the transplanted hepatoma 31 and the normal and regenerating livers of rats (*1–5, 9*), the present studies of rat fetal liver provide a picture of the comparative activity of many enzyme systems in four homologous tissues of a single species of animal, the rat.

EXPERIMENTAL PROCEDURE

Pregnant Osborne-Mendel rats furnished the embryos for this study. Transplants of hepatoma 31 and the normal and regenerating livers with which the previous enzymatic studies were performed were also obtained from Osborne-Mendel rats. The age of the embryos varied from 1 to 2 weeks; the fetal livers obtained from the embryos were pooled in all cases. The extraction procedures have been described (*1*). The conditions for the determination for the activity of each of the enzymes were followed exactly as before; the enzymes investigated included arginase (*10*), catalase (*11*) xanthine dehydrogenase (*11*), acid and alkaline phosphatase (*12*), ribonucleodepolymerase (*5*), thymonucleodepolymerase (*13*), amylase (*11*), cystine oxidase (*4*), and the urea-synthesizing systems (*2*). The method of representing the activity of each of the enzymes is also given in the foregoing references. Data on rabbit fetal liver have been obtained on

TABLE 1.—*Enzymatic activity of normal adult, regenerating, fetal, and neoplastic hepatic tissues of the rat* [1]

Tissue	Enzyme											
	Arginase (Q)	Catalase [2]	Xanthine dehydrogenase	Acid phosphatase (Q acid)	Alkaline phosphatase (Q alkaline)	Ribonucleodepolymerase [3]	Thymonucleodepolymerase	Amylase [4]	Urea synthesis [5]	Cystine oxidase [6]	Cytochrome oxidase [7]	*d*-Amino acid oxidase [7]
			Minutes									
Normal adult liver	213	1.7	14	25	4	0.09	6.6	1.5	0.14	9.0	30.9	0.24
Regenerating liver [8]	250	1.7	27	29	3	.08	6.3	1.6	.13	9.1	30.1	.19
Fetal liver	60	.06	[9] >300	48	27	.08	5.8	1.5	0	4.5	12.3	.035
Transplanted hepatoma 31 [10]	21	.002	28	52	542	.07	5.3	1.5	0	0	6.8	.022

[1] All data on Osborne-Mendel rats.
[2] Measured in cubic centimeters of O_2 per second.
[3] In milligrams P per cubic centimeter in 2 hours.
[4] In cubic centimeters 0.02 N thiosulfate in 2 hours.
[5] Urea N per cubic centimeter in 2 hours.
[6] Mols NH_3 N x 10^6.
[7] Cubic millimeters O_2 per milligram per hour. Data of Shack (7).
[8] Obtained 48 hours after excision of 60 percent of the liver.
[9] Since the data refer to the decolorization time of methylene blue, the greater the time given, the weaker the enzyme activity.
[10] Originally induced by feeding *p*-dimethylaminoazobenzene.

all but the two last-mentioned systems (*1, 5*).

No data have been given in the earlier references for the activity of cystine oxidase or for the synthesis of urea in regenerating rat liver. Such data have have since been obtained on tissue supplied through the kindness of Dr. Eschenbrenner and have been incorporated into the present paper. These data, together with those on the fetal rat liver, are given in table 1. Included for comparison are the data obtained earlier (*1–5*) on the normal and regenerating adult liver and on the transplanted hepatoma 31, as well as data by Shack (*9*) on the activity in these tissues of two different oxidases. The activity of each enzyme is given in table 1 under identical experimental conditions for each of the four kinds of homologous rat tissues. Each determination was made on at least three different samples of tissue. The variations were never more than 20 percent and often less.

DISCUSSION

Normal and Regenerating Adult Livers

With the exception of xanthine dehydrogenase, the values for the activity of all the enzymes tested are nearly identical in normal and regenerating adult livers. Xanthine dehydrogenase is lower in activity in the regenerating liver, a fact that may have some correlation with the finding (*14*) that nucleic acid is synthesized at a rate higher in this tissue than in normal liver. There is more than sufficient flavine in the regenerating liver (*15*) to supply the prosthetic group of xanthine dehydrogenase, and the lowered value for this enzyme must therefore be due to some alteration in the protein moiety of this enzyme.

Fetal and Adult Liver

Fetal liver apparently possesses little or no xanthine dehydrogenase activity and little or no capacity to synthesize urea. Although arginase, catalase, and cystine, cytochrome, and *d*-amino acid oxidases are present, their activity is considerably less than that observed in the adult liver. The activity of both acid and alkaline phosphatase is greater in fetal than in adult liver and may be due to the osteoplastic activity of the embryo. The activity of both ribonucleodepolymerase and thymonucleodepolymerase and of amylase (phosphorylases) is nearly identical in fetal and in

adult liver. The role of the depolymerases has been discussed before (*5*). The relation of the activity of fetal rabbit hepatic tissue to the adult rabbit liver previously observed (*1, 5*) is in general the same as that observed with the comparable rat tissues.[1]

Hepatoma 31 and Adult Liver

When the rat hepatic cell becomes neoplastic, certain enzymes decrease in activity (arginase, catalase, xanthine dehydrogenase, urea-synthesizing systems, and cystine, cytochrome, and *d*-amino acid oxidases), other enzymes increase in activity (acid and alkaline phosphatases), while still others apparently remain the same (ribonucleodepolymerase, thymonucleodepolymerase, and amylase).[2] Since these comparisons have been discussed in earlier papers (*1–5*), no further emphasis is necessary here except to point out the similarity in the enzymatic relations between the neoplasm and adult liver on the one hand and between fetal liver and adult liver on the other.

Fetal Liver and Hepatoma 31 Compared With Normal and Regenerating Liver

The enzymatic activity of fetal liver and of the hepatoma appear to run parallel when compared with the activity of normal and regenerating liver. When the activity of any one enzyme in the hepatoma is less than, greater than, or equal to the activity of the same enzyme in normal adult or regenerating liver, so too is the activity of that enzyme, respectively, in the fetal liver. Thus, in category 1, arginase, catalase, xanthine dehydrogenase, the urea-synthesizing systems, and cystine, cytochrome, and *d*-amino acid oxidases are all lower in activity in both fetal liver and the hepatoma than in normal or regenerating liver; in category 2, acid and alkaline phosphatases are both higher in activity in fetal liver and the hepatoma than in normal or regenerating liver; whereas in category 3, ribonucleodepolymerase and thymonucleodepolymerase and amylase are nearly identical in all four tissues. It would appear, therefore, that of the hepatic tissues considered, normal and regenerating liver form one class, fetal liver and the hepatoma a second class.

What have the enzymes of each category in common? In category 1, arginase, catalase, xanthine dehydrogenase, and cytochrome and *d*-amino acid oxidases are known to be conjugated proteins. Xanthine dehydrogenase and *d*-amino acid oxidase contain riboflavin as the prosthetic group, arginase contains manganese, whereas catalase and cytochrome oxidase contain iron. Whether cystine oxidase contains a prosthetic group is not known at the present time. In fetal liver or in the hepatoma, apparently, the proper conditions for conjugation are not present, owing possibly to either incomplete development or alteration of the protein moiety involved. In category 2, the state of acid and alkaline phosphatases is not yet known because neither enzyme has been sufficiently purified. In category 3, only ribonucleodepolymerase has been obtained in a purified condition. This enzyme is a relatively simple protein of low molecular weight. The mode of action of the three enzymes in this category is that of a depolymerization of high-molecular-weight sub-

[1] *Fetal liver presents a certain complication, inasmuch as this tissue contains blood-forming cells which might be expected to dilute the activity of the hepatic elements. Since neither the extent of this possible dilution nor the contribution to the activity of each enzyme by the hematopoietic elements themselves is known, it is not feasible to discuss this matter further.*

[2] *The phenomenally high value for the alkaline phosphatase activity of the transplanted hepatoma 31 has been confirmed by independent sources on primary tumors induced in rats by the feeding of* p-*dimethylaminoazobenzene* (16).

strates. This property of attacking large molecules sets off the enzymes of this category from those of categories 1 and 2. Whether this is the reason why the enzymes of category 3 in fetal liver do not change in activity when the embryo develops into the adult or when the adult liver regenerates or becomes neoplastic cannot be answered at the present time. The facts simply indicate that the activity of these enzymes (category 3) is set at a certain value in the liver of the fetus by at least half the term of intra-uterine development and remains at this value independent of maturity or of later neoplastic change of the liver.

SUMMARY

The activity of several enzyme systems in fetal rat livers was determined, and the data obtained were compared with those on the transplanted hepatoma 31 and on normal adult and regenerating livers in rats. The results indicated a similarity of the relation of fetal to adult liver on the one hand and of the hepatoma to adult liver on the other.

Certain enzymes (arginase, catalase, xanthine dehydrogenase, urea-synthetic systems, and cystine, cytochrome, and *d*-amino acid oxidases) were lower in both fetal liver and hepatoma than in adult liver; others (acid and alkaline phosphatases) were higher in both fetal liver and hepatoma than in adult liver; still others (ribonucleodepolymerase, thymonucleodepolymerase, and amylase) had the same activity in all four kinds of hepatic tissue. Thus, the properties of the hepatoma and and of fetal liver ran parallel in relation to those of the adult liver in every case.

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Range in Activity of Several Enzymes in Normal and Neoplastic Tissues of Mice

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It has long been known that the activity of a particular enzyme may vary considerably in the normal animal from one tissue to the other. For example, catalase is very active in the liver but very weak in muscle, and alkaline phosphatase is very active in the intestine but weak in the liver. Under normal conditions, each tissue carries out definite and specific functions, which are based upon the possession by the tissue of a unique pattern of enzymic catalysts. Every normal tissue can, therefore, be characterized by this pattern.

Does the activity of each enzyme in tumors of various origin also differ considerably from tumor to tumor? As a partial answer to this question, the range in activity of several well-defined enzyme systems has been examined under identical experimental conditions in available normal and neoplastic tissues of a single species of animal, the mouse. No attempt is made to call this study in any way complete. It is obvious that not every normal tissue, not every conceivable tumor, and not every enzyme system have been investigated. Since the tumors studied arose in animals of various strains, care has been taken to ascertain whether the enzymatic activity of the normal tissues differed from strain to strain. However, the validity of the present findings is limited to the particular normal and neoplastic tissues investigated, to the particular enzymes studied, and to the particular species and strains of animal employed.

EXPERIMENTAL PROCEDURE

The enzymes included catalase, arginase, xanthine dehydrogenase, acid and alkaline phosphatase, thymonucleodepolymerase, and ribonucleodepolymerase. The technique of measuring the activity of these enzymes has been described in several papers in this Journal (*1–17*). Each enzyme, whether examined in normal or in neoplastic issues, was studied under identical experimental conditions, which included the use of tissue extracts at equal concentrations of total nitrogen. Several samples of each tissue were studied; in the case of each enzyme the determination of activity varied by no more than ± 10 percent from tissue to tissue, and the reproducibility of the determinations for any one tissue was well within this limit. References to the tumors employed, with the exception of the transplanted rhabdomyosarcoma (*18*), have been made in the foregoing series of papers. Part of the data given in the present communication has been taken from the earlier papers.

Since the tumors arose in mice of various strains, chiefly A, C, C3H, and Dilute Brown, it was desirable to know whether the activity of the same tissue differed from strain to strain. It was found that no strain difference apparently existed for any of the enzymes in any of the tissues of these strains of mice. The collected data are given in table 1.

TABLE 1.—*Enzymatic activity in tumors and in normal tissues of mice* [1]

Tumors	Strain	Arginase (Q)	Catalase [2]	Xanthine dehydrogenase	Acid phosphatase (Q acid)	Alkaline phosphatase (Q alkaline)	Thymonucleodepolymerase	Ribonucleodepolymerase [3]
				Minutes				
Hepatoma 1 [4]	Dilute Brown × I	40	0.6	10	10	1	11.1	
Hepatoma A	C3H	34	.05	38	21	0	21.3	0.26
Hepatoma 98/15	C3H	30	.08	35	22	1	20.6	.30
Hepatoma 7A/77	A	37	.8	18	22	4	15.4	.29
Hepatoma 587	A	33	.24	20	21	5	15.2	.27
Mammary tumor [5]	C3H	114	.01	30	21	22	19.5	.27
Lymphoma 72942 [4]	A	26	.007	25	10	10	3.9	.17
Lymphoma Y103	Dilute Brown	31	.007	40	12	4	4.2	.11
Melanoma [4]	do	49	.008		19	1	3.0	.09
837 [4]	do	49	.003	15	23	2	3.4	.16
CR 180 [4]	do	49	.003	15	21	2	3.4	.15
Intestinal adenocarcinoma [4]	A	26	.001	30	19	3	14.4	.20
Squamous-cell carcinoma of stomach [6]	C57 Brown	52	.001	17	6	3	3.2	.16
Methylcholanthrene-induced sarcoma [6]	C3H	25	.0	22	19	2	4.5	.12
Rhabdomyosarcoma [4]	C	28	.003	42	8	24	15.2	.18
Normal tissues	C, C3H, A, Dilute Brown							
Liver	C, C3H, A, Dilute Brown	246	8.0	10	12	4	14.3	.12
Hyperplastic breast [7]	C3H, Dilute Brown	67	.02	45	18	9	8.0	.04
Lymph nodes	C3H, A, Dilute Brown	20	.02	240	49	8	[8] 25	.46
Bone marrow	A, Dilute Brown	4	.01	45	22	23	7.3	.60
Spleen	C, C3H, A, Dilute Brown	6	.12	30	73	17	16.1	.28
Kidney	do	42	.8	15	15	1,072	10.2	.08
Muscle	do	4	.005	92	19	2	12.0	.07
Skin	do	27	.006	45	30	5	10.0	.17
Lung	do	50	.22	>300	33	36	8.2	.06
Intestinal mucosa	do	80	.0	6	34	2,789	14.7	.68
Gastric mucosa	do	4	.0	>300	27	17	6.1	.27
Thymus	C3H, Dilute Brown	2	.001	>300	5	3	3.1	.12
Pancreas	C, C3H, A, Dilute Brown	8	.01	30	10	1	5.4	.78

[1] Experimental technique and methods of representing activity are given in the following references: arginase (*1*), catalase (*3*), xanthine dehydrogenase (*3*), phosphatases (*7*), thymonucleodepolymerase (*6*), ribonucleodepolymerase (*17*).

[2] Activity in cubic centimeters of O_2 per second.

[3] Increase in acid-soluble P in milligrams per cubic centimeter in 2 hours.

[4] Transplanted.

[5] Spontaneous.

[6] Primary tumor induced by subcutaneous implantation of methylcholanthrene.

[7] Induced by subcutaneous implantation of stilbestrol.

[8] Probably slightly higher than value given.

ARGINASE

The range in activity of arginase in the tumors studied is 25–114. The value of 114 is that of the spontaneous mammary tumor and is considerably beyond the median value of the rest of tumors. If this value is excepted, the range becomes 25–52, which is rather narrow.

In the normal tissues, the values for the activity of this enzyme range from 2 to 246. The highest value is that of the liver in which arginase is relatively concentrated. The urea cycle, in which arginase participates, is found exclusively in the liver. If the value for the liver activity is excepted from the consideration of the normal tissues, the range becomes 2–80, which is still greater than that of the tumors, even when the value of the mammary tumor is taken into account.

With the exception of the mammary tumor, the values for all the tumors appear to cluster about a mean value of about 35 ± 15. No such clustering appears in the values of the normal tissues, for these vary considerably; some of the normal tissues possess an activity higher and others less than that of the median value of the tumors.

CATALASE

Among the tumors, the catalase activity varies from 0 to 0.8. The highest values are observed among the transplanted hepatomas, which is an indication that these tumors still reflect some of the character-

istics of the liver tissue from which they originated. In the other tumors, the enzyme is present only in traces, and even the traces may be owing to the presence of some residual blood.

Among the normal tissues, the liver is highly active and this fact partially accounts for the considerable range in this enzyme of 0–8.0. If the value of the liver is excepted, the range becomes 0–0.8, which is that of the tumors. If the values of the liver and of the hepatomas are omitted from the comparisons, the range in activity among the normal tissues is still considerably greater than among the tumors, since some of the normal tissues, like the kidney and the spleen, are fairly active whereas other tissues, like mucosa, are completely lacking in the enzyme.

Xanthine Dehydrogenase

The range in xanthine dehydrogenase activity among the tumors is 0 to 42. The median value may be taken at about 27 ± 17. In this connection, it must be recalled that since these figures refer to the decolorization time of methylene blue in the digests, the lower values refer to the more active tissues. In contrast with the relatively narrow range among the tumors, the normal tissues vary in value from 6 to >300, with considerable variation from tissue to tissue.

Acid Phosphatase

The range in acid phosphatase activity among the tumors is 6 to 23, and among the normal tissues 5 to 73. The extent of these ranges, while greater among the normal tissues than among the tumors, is not so strikingly different as that observed for the enzymes previously described. A roughly chosen median value for the tumors might be 15 ± 9, and for the normal tissues 40 ± 30.

Alkaline Phosphatase

Among the tumors, the range in alkaline phosphatase activity appears to be 0 to 24, most of these tissues possessing little activity. The range here is relatively narrow. Among the normal tissues, the variation is considerable, the values ranging from 1 to 2,789. The highest values are observed in intestinal mucosa and in the kidney. If these values are excepted, the values for the remaining normal tissues are low, and of the magnitude of those found among the tumors.

Thymonucleodepolymerase

Thymonucleodepolymerase, which accomplishes the catalytic depolymerization of thymus nucleic acid, possesses a range among the tumors of 3.0 to 21.3, and among the normal tissues of 3 to 25. Neither range is considerable, and both are of the same order of magnitude.

Ribonucleodepolymerase

Ribonucleodepolymerase, which accomplishes the catalytic depolymerization of yeast nucleic acid, possesses a range among tumors of 0.09 to 0.30, and among the normal tissues of 0.04 to 0.78. The range is greater among the normal than among the neoplastic tissues.

Homologous Tissues

Comparisons have been made thus far, on the one hand, of all the normal tissues, and on the other hand, of all the tumors. If now the comparisons are made among only those tumors for which the normal tissue analogues are available, the following pairs of homologous tissues must be considered: (1) Liver and hepatomas; (2) lymph nodes and lymphomas; (3) breast and mammary tumors; (4) intestinal mucosa and adenocarcinoma; and (5)

muscle and rhabdomyosarcoma. The range in activity for each enzyme among the normal tissues of this group and the neoplasms derived from them may be ascertained from table 1. It is clear that the same conclusions hold when the groups of homologous normal and neoplastic tissues (table 1) are considered as when all the tissues, without regard to this limitation, are considered, namely, that with the exception of thymonucleodepolymerase, the range in each of the enzymes is much greater among the normal tissues than among the tumors.

Pertinent Data From Other Sources

Shack (*19*) estimated the cytochrome oxidase activity of most of the mouse tumors described in table 1. The range in values of Q, as Shack defined Q, was 3.0 to 7.4. No comparable studies are available on various mouse normal tissues, hence exact comparisons of the range of activity of this enzyme in normal and neoplastic tissues of mice must await further investigation. There are, however, some data by Stotz (*20*) and by Schneider and Potter (*21*) on the activity of cytochrome oxidase in several normal tissues of the rat; the values of the activity units defined, respectively, by Stotz and by Schneider and Potter range from 1.3 and 109 for lung, to 9.7 and 1,699 for heart muscle. Although it is highly probable that the range in the activity of this enzyme is nearly the same in normal rat and mouse tissues, the comparison of this range in neoplastic tissues of mice and normal tissues of rats must be made with all due reservations. If such a comparison be made, however, it is apparent that the range in activity among the former is considerably less than among the latter tissues. Both Stotz and Schneider and Potter included the study of two or three rat tumors, of which the range in activity was much narrower than among normal tissues; but the number of tumors was too few and the types available were not sufficiently varied to provide adequate comparisons.

Taylor, Pollack, and Williams (*22*) observed that the range in the total vitamin B content of a large group of tumors of various origin was much narrower than that among normal tissues. Although their conclusion that "malignant neoplasms of various types and from various animals tend to have similar cellular metabolism, forming in effect a common tissue type" may not be quite warranted at the present time, it is interesting that various types of components, such as vitamins and enzymes, are quantitatively more alike among tumors than among normal tissues.

Question of Etiology

Neoplasms may arise in animals either spontaneously or by induction through a wide variety of unrelated extrinsic agents. Among these agents may be various hydrocarbons, dyes, viruses, and forms of radiation. Certain tumors which appear to originate spontaneously may actually require an extrinsic factor or agent. Is the mechanism of carcinogenesis independent of the nature of the causative agent, whether the latter be extrinsic or intrinsic in origin? This question cannot be answered at the present time. On the other hand, it is possible to say, on the basis of the present data, that the ultimate properties of the tumor itself appear to be independent of the nature of its origin. As an example, the mouse hepatomas may be cited. The available data include studies on hepatoma 1 in I×Dilute Brown mice (F_1 generation) originally induced in I mice by injection of 2-amino-5-azotoluene, hepatoma A in C3H mice originally induced by injection of aminoazotoluene, he-

patoma 7A/77 in A mice originally induced by injection of carbon tetrachloride, hepatoma 98/15 which arose spontaneously in a C3H mouse, and hepatoma 587 which arose spontaneously in an A mouse. We have, therefore, hepatomas in two different strains which arose spontaneously; hepatomas in two different strains which were induced by the same chemical carcinogen, aminoazotoluene; and two hepatomas in each of two strains, one of which was induced by a chemical carcinogen whereas the other arose spontaneously. The data in table 1 indicate that the enzymatic properties of all these hepatomas, irrespective of the origin of the latter, are nearly the same. The cytochrome oxidase values of Shack (*19*) are also nearly the same for these hepatomas. In connection with the data on the other tumors in table 1, it would appear that no matter what or where the origin of a tumor may be, it ends by possessing a set of properties nearly common with all other tumors.

Osteogenic Sarcoma, a Possible Exception

Enzymatic studies on the transplanted Barrett osteogenic sarcoma 1 (*23*) revealed values for the following enzymes (see table 1 for units): arginase, 27; catalase, 0.001; xanthine dehydrogenase, 35; thymonucleodepolymerase, 3.1; and ribonucleodepolymerase, 0.10. These values are well within the limits observed for the other tumors described (table 1). On the other hand, the phosphatase values are, as might be expected, phenomenally high, the acid phosphatase being 135 and the alkaline phosphatase 1,100. These values are far outside the range for the phosphatases of any of the tumors studied. The acid phosphatase of the osteogenic sarcoma exceeds that of any of the normal tissues, and the alkaline phosphatase value is equaled only by that of the kidney and is exceeded only by that of the intestinal mucosa.

A high phosphatase activity is always associated with osteoblastic activity (*15*). Since this activity represents a specialized structural function, the osteogenic sarcoma has not been included in table 1. At the present time, the relation of the phosphatase activity of the osteogenic sarcoma to that of the normal osteoblastic cells is not known. To clarify this point, the activity of the epiphysis of normal growing bone should be determined; in the case of mice this determination would be rather difficult.

DISCUSSION

With one exception, the range of the enzyme systems studied is considerably greater among the normal than among the neoplastic tissues. The exception, that concerned with thymonucleodepolymerase, is of interest because this enzyme is very probably an exclusively nuclear component (*17*). When a normal tissue becomes neoplastic, e. g., nodes converted to lymphomas, or liver to hepatomas, this enzyme may either increase or decrease in activity, but the change is relatively small compared with the changes in the activity of other enzymes. It is partly for this reason, and partly because the various tissues do not differ greatly among themselves in the value for this enzyme, that the range in activity of the thymonucleodepolymerase is rather narrow and rather similar in normal tissues and in tumors. Apparently, the nuclear activity in all kinds of tissues maintains a level which does not vary greatly from tissue to tissue.

The explanation for the much narrower range among the other enzymes in the tumors as contrasted with the normal tissues is not far to seek. To a very large extent it may be based upon the severe decrease in certain of the specific functional activities

of a tissue when the tissue becomes neoplastic (*15*). The data in table 1 indicate the considerable drop in activity of hepatic arginase and catalase, of intestinal alkaline phosphatase, and of lymphatic ribonucleodepolymerase when these tissues become neoplastic. To this evidence may be added the similar loss in gastric pepsin and rennin (*9*), the hepatic urea cycle (*13*), cystine oxidase (*16*), and muscle creatine [1] when the gastric mucosa, liver cells, and muscle become adenocarcinoma, hepatoma, and rhabdomyosarcoma, respectively. On the other hand, certain enzymes, such as hepatic acid phosphatase and mammary arginase, increase in activity when the normal tissues become neoplastic, but this increase in no case is as great in magnitude as the loss encountered in other enzyme systems.

It would appear that the activity of each of the enzymes in the tumors, with the exception of catalase in the hepatomas, is more nearly of the same order of magnitude than is the activity of each of the enzymes in the normal tissues. This may mean that when a normal tissue becomes neoplastic, it tends to lose in large degree certain of its specific functions; and the neoplasm, while it may retain some vestige of the activity of the tissue of origin (as in the case of hepatic catalase), resembles other tumors more than it does the tissue of origin. The tumors possess a more nearly common enzymatic level, so that from this standpoint the tumors are more nearly like each other than they are like normal tissues or than normal tissues are like each other.

[1] *The creatine content of normal mouse muscle, determined by methods described earlier* (12) *has been found to be 375 mg. per 100 gm. of wet tissue. By the same methods the creatine content of the transplanted rhabdomyosarcoma in C mice* (18) *has been found to be so low as to be unmeasurable.*

SUMMARY

The activity of arginase, catalase, xanthine dehydrogenase, acid and alkaline phosphatase, thymonucleodepolymerase, and ribonucleodepolymerase was determined in the following normal tissues: liver, breast, nodes, marrow, spleen, kidney, muscle, skin, lung, intestinal and gastric mucosa, thymus, and pancreas, and in the following tumors: hepatomas 1, A, 98/15, 7A/77, and 587, spontaneous mammary lymphomas 72942 and Y103, melanoma, S37, CR 180, intestinal adenocarcinoma, squamous-cell carcinoma of the stomach, sarcoma induced by methylcholanthrene, and rhabdomyosarcoma. All tissues were obtained from mice.

The range in activity for each of the enzymes, with the exception of thymonucleodepolymerase, was much greater among the normal tissues than among the tumors. In the case of the exception noted, the range was very nearly the same for both normal and neoplastic tissues. The difference in the ranges of the enzymes in normal tissues and in tumors has been explained on the basis of the severe decrease observed in many of the specific functional activities of certain tissues when these tissues became neoplastic.

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556830—43——3

Note on the Liver Catalase Activity of Pregnant Mice and of Mice Bearing Growing Embryonic Implants

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The liver catalase activity of mice and rats (*1*)[1] and of human beings (*2*) bearing a wide variety of neoplasms is considerably depressed below the normal value. The effect on this liver function is (1) progressive with and proportional to the growth rate of the tumor, and (2) reversible on surgical removal or regression of the tumor. The mechanism of this depression of the liver catalase activity of tumor-bearing animals is not fully understood at the present time. In view of the appreciable degree of anemia which generally accompanies neoplastic growth (*3*) and because both hemoglobin and catalase possess the same hemin prosthetic group, it has been suggested (*4*) that the tumor in some way interferes with the normal synthesis of the hemin prosthetic group.

The question arises whether depression of the liver catalase activity would occur in the presence not only of malignant but also of benign growths. In other words, is the effect due to any growing tissue within the host, or may it be specifically related to the fact that the growing tissue is malignant in nature? As a partial answer to these questions, the liver catalase activity was determined both of pregnant mice and of mice bearing progressively growing, subcutaneously implanted embryonic tissue mash which was originally obtained from 2-week-old embryos of the same strain as the animals subsequently inoculated. The mash grew progressively for several months. The liver catalase activity of pregnant mice and of mice bearing embryonic implants was found to be normal, and hence in the animals studied these benign growths produced no effect comparable with that of tumors. The data are given in table 1.

TABLE 1.—*Liver catalase activity of pregnant mice and of mice bearing embryonic implants* [1]

Strain	Condition	Concentration of liver extract [2]	Liver catalase activity [3]
C3H	Normal	0.6	2.0
		.3	.8
C3H	Pregnant 2 weeks	.6	2.0
		.3	.7
C3H	Bearing embryonic implant 3 weeks.	.6	2.0
		.3	.9
C3H	Bearing embryonic implant 5 weeks.	.6	1.9
		.3	.8
C3H	Bearing embryonic implant 2 months.	.6	2.1
		.3	.7
Dilute Brown	Normal	.6	1.9
		.3	.8
Do	Pregnant 2 weeks	.6	2.0
		.3	.9
Do	Bearing embryonic implant 2 weeks.	.6	1.8
		.3	.8
Do	Bearing embryonic implant 4 weeks.	.6	2.0
		.3	.9

[1] Embryo mash obtained from embryos 2 weeks old.
[2] Milligrams N per cubic centimeter.
[3] Cubic centimeters O_2 per second.

[1] *This paper refers to previous papers in this series of studies.*

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Effects of Feeding Heated Lard to Rats, With a Histologic Description of the Lesions Observed

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INTRODUCTION

Available statistics indicate that cancer of the digestive tract in man accounts for approximately 45 percent of all deaths from cancer at the present time. It seems logical to suspect dietary constituents of being involved in the genesis of this disease, since food and its digestive products come into intimate contact with the tissues lining the alimentary canal.

Many investigators have attempted to produce gastric neoplasms in experimental animals by dietary means. In 1913 Singer (*1*) described the production of gastric lesions in a high percentage of his experimental rats and tentatively ascribed an infectious origin to these lesions. Apparently unsuspected by Singer, the nutritionally poor diet which he fed his animals may have been an important factor. Similarly, the lesions in the stomachs of nematode-infested rats described by Fibiger (*2*) are of uncertain etiology. In repeating Fibiger's experiments, Passey, Leese, and Knox (*3*) found that rats maintained on Fibiger's diet of white bread and water developed some gastric lesions even in the absence of nematode infestation, although these lesions were less severe than those seen in a similar group infested with *Gonglyonema neoplasticum*. Other rats carrying the nematode, on a complete diet, exhibited but insignificant changes in the gastric muscosa. Bullock and Rohdenburg (*4*) attempted to demonstrate that Fibiger's results were caused by nonspecific chronic irritation. They resorted to the use of mechanical and chemical irritants and obtained results similar to but not identical with those of Fibiger.

Pappenheimer and Larimore (*5*) reported experiments which indicated for the first time that dietary deficiencies might be a factor in the etiology of gastric lesions. They were unable to ascertain the exact nature of the deficiency in their experimental diets. Subsequently, a number of investigations dealing with the role of dietary deficiency in gastric lesions have been reported. Detailed accounts may be found in the recent work of Klein and Palmer (*6*), Beck and Peacock (*7*), Morris and Lippincott (*8*), and Brunschwig and Rasmussen (*9*).

Since the evolution of our present knowledge of chemical carcinogens, several investigators have described experiments designed to demonstrate a relationship between dietary fats and gastric cancer. Roffo (*10*) reported the production of adenocarcinoma of the glandular portion of the stomach and "fuso-cellular sarcoma" of the liver in rats fed heated fats; Domagk (*11*) found tumors in the stomachs of mice fed a diet rich in olive oil; Waterman (*12*) claimed that he obtained malignant tumors in the stomachs of mice fed cholesteryl oleate; Veldstra (*13*) offered Δ 3,5-cholestadiene as a possible link between heated

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dietary fats and gastric neoplasia; and Widmark (*14*) concluded that extracts of roasted foods (coffee, butter, horse muscle) contain one or more carcinogenic substances. The chemistry involved in the experimental work cited was analyzed by Cook and Kennaway (*15*) and the pathology by Klein and Palmer (*6*). Recently, Steiner, Steele, and Koch (*16*) investigated the possible carcinogenicity of drastically heated meat fats, cholesterol, sesame oil, and acrolein. The feeding and subcutaneous injection of these heated substances led to essentially negative results.

Roffo (*10, 10a*) fed rats bread and milk to which was added an equal amount of lard, beef tallow, mutton tallow, or olive oil, which had been heated to 350° C. for one-half hour. Beck and Peacock (*7*) fed rats and mice similarly heated fats incorporated into an adequate diet. They were unable to find any evidence of malignant glandular tumors or of sarcomas. Papillomatosis was found in the forestomach of animals fed heated-fat diets and was attributed to an "anti-vitamin A" factor in the heated fats.

The temperature employed by Roffo was much higher than that used in ordinary cooking procedures, nevertheless, his observations appeared to be of sufficient interest to warrant further study. The hypothesis that merely heating a major dietary component resulted, presumably, in the formation in that foodstuff of substances carcinogenic, at least for the rat, raised several questions: (1) Could Roffo's claim that the heating of fat converted cholesterol or other sterol into a carcinogenic substance be verified either chemically or biologically? (2) Could gastric neoplasms be produced with regularity by feeding rats a diet rich in heated fat? (3) Could prolonged heating of fats at temperatures lower than those used by Roffo produce the same effect on the stomach of the rat?

The present paper reports the results obtained by feeding heated lard to rats until they were dying, and the gross and microscopic examination of the stomach, liver, and certain other affected tissues. Simultaneously, a chemical study was made on lard that had been heated under varying conditions, with the purpose of clarifying the nature of the chemical changes that occur when lard is heated at extreme temperatures.

In the original plan of our work, it was tentatively proposed to study the effects of heating three animal fats (pork, beef, mutton) and one vegetable fat consisting for the most part of a partially hydrogenated cottonseed oil. The physical equipment and technical assistance for studying these four fats simultaneously was not available. Therefore, because of the extensive use of lard in the human dietary, it was decided to study lard first. An examination of Roffo's protocols indicated that his results were much the same, regardless of the fat used. Any degree of severity or difference in time of onset of the stomach lesions was not apparent in the groups of rats fed the four heated fats in his experiments. Hence, it seemed desirable to us to concentrate on just one fat and to study the effects of variations of time and temperature of heating.

MATERIALS AND METHODS

Three-month-old Wistar rats, equally divided as to sex and selected at random, were used. They were placed on the special high-fat diets after the period of most rapid growth had occurred. These rats were of the same approximate age as the animals used in the extensive experiments described by Roffo (*10*). The animals were housed individually in wire-cloth-bottom metal cages. Individual records of food intake and body weight were made at weekly intervals throughout the experiment. Daily observation was

made of each animal, and toward the end of the experiment moribund animals were killed by gas and immediately autopsied. In spite of every precaution, some animals died at night and autopsy was not performed for some hours after death. In such cases, if the animal had undergone extensive autolysis, it was not included in the interpretation of the histologic material.

The temperature of the animal room was maintained at 80° F. during the period of the year when heat was available. During a few hot days in summer the temperature rose occasionally to 88° to 90° for short periods. Since the animals were in wire-cloth-bottom cages, they did not appear to become overheated on such occasions.

The cages were washed and sterilized in boiling water followed by live steam, at frequent intervals. No nesting material was provided for the first 18 months, and during this period many animals became excessively greasy. It is believed that this condition was brought about by the inability of the rats to groom themselves properly after getting fat smeared on their paws while eating the high-fat diet. Some loss in food probably occurred in this way which could not be taken into consideration in the food-intake records. After about 18 months crepe-paper shavings were placed in all cages, and it was observed that the greasy condition of the animals' hair soon disappeared. At the same time the bedding material became oil-soaked and had to be removed frequently. This was not due to actual spillage of food but resulted from food that adhered to the feet and hair of the animals.

EXPERIMENTAL PROCEDURE

The lard was homogeneously rendered under carefully controlled conditions and was of a quality equal or superior to the best grades of market lard. It was stored in hermetically sealed cans at −10° C. After 2 years samples of this lard showed no significant changes in physical or chemical characteristics.

The lard was heated only as needed for the experimental diet. Stored lard, 3 pounds at a time, was warmed to the molten state on the water bath and poured into either a pyrex beaker or a glazed, 4-l., Coors porcelain beaker and heated on a heavy-duty hot plate. Thirty, forty-five, and sixty minutes were usually required to reach temperatures of 200°, 300°, and 350° C., respectively. After the desired temperature was reached, it was maintained within a range of ±3°. At temperatures above 330°, the rate of thermal decomposition of the lard was accelerated and fuming became severe. Preliminary lots were prone to catch fire spontaneously. In order to prevent this, the heating vessel was covered with a galvanized-sheet-iron plate, which was notched for insertion of a thermometer. This plate served to prevent contact of atmospheric oxygen with the volatile fumes at or above their flash point. After the desired temperature was maintained for the required time, the lard was allowed to cool until it could be conveniently handled (200°–250°), poured into suitable containers, and returned to the cold room. Representative samples of the heated lard were frequently set aside for immediate chemical study.

Preliminary exploratory experiments revealed that our rats were loath to eat and were unable to survive more than a few weeks on a diet containing 50 percent of lard that had been heated at 350° C. for one-half hour. Lard heated under these conditions was found to have an unpleasant taste and odor; when heated at lower temperatures it appeared to be as palatable as unheated lard. Because of the relative

paucity of information in the literature concerning the chemical changes induced in severely heated edible fats, it was necessary to study the nature of the decomposition. The results of the studies made on the unheated stock lard and on samples heated from 200° to 250° for varying periods are reported elsewhere (*18*).

The diets, which were prepared at 2-week intervals, were stored in glass vessels in a cold room maintained at −5° C. and used as needed. Fresh diet was given the animals in clean, dry, glass feeding cups once a week, and sufficient food was given to last an entire week. With the technical facilities available, it was not possible to feed the animals at more frequent intervals. All diets were isocaloric so that the food consumption should be approximately the same and comparable for all groups of animals.

The basal portion, comprising 50 percent of the complete diet, contained the following substances:

	Percent
Ground whole wheat	22.35
Skim milk powder	24.00
Brewers' yeast (debittered)	2.00
Corn oil, fortified with vitamin A ester [1] and calciferol [2]	[3] 1.00
Sodium chloride	.50
Ferric citrate	.15
Total	50.00

[1] Vitamin A ester was supplied by Distillation Products, Inc., Rochester, N. Y.
[2] Calciferol was supplied by Mead Johnson Co., Evansville, Ind.
[3] Equivalent in vitamin A and D to 2 percent U. S. P. XI cod-liver oil.

The complete diets, which consisted of the basal portion plus added lard, are listed in table 1. The protein content of the diet was roughly 11.5 percent. The wheat was a soft winter variety and therefore of low protein content. With the addition of yeast and vitamins, this diet was probably of a higher nutritive quality than the bread and milk diet Roffo fed his rats. It is believed that the complete diet, containing 50 percent of unheated lard, was satisfactory for the adequate nutrition of the rat over an experimental period of more than 20 months. The control animals were able to increase in body weight throughout this period and showed no gross clinical evidence of nutritional deficiency.

TABLE 1.—*Diets used in experiments with heated lard*

Diet No.	Fat component	Treatment		Proportion of diet
		Temperature of heating	Time of heating	
		°C.	*Minutes*	*Percent*
71	Lard	None	None	50.0
72	do	300	30	50.0
73	do	350	30	35.0
	do	None	None	15.0
74	do	300	120	50.0
75	do	None	None	38.0
	Free fatty acids from unheated lard	None	None	12.0

Each group contained 30 animals equally divided as to sex, except the group on diet 75, which consisted of only 20 animals. Figure 1 gives the average body-weight curves of the female rats for the first 75 weeks of the experiment. As there was no essential difference between males and females, only 1 set of body-weight curves is presented. In the group of rats fed diet 74, which contained lard heated to 300° C. for 2 hours, the average body weight decreased for 2 weeks at the start of the experiment, after which the body weight was just maintained for about 4 months; then there was a gradual decrease in weight to the end of the experiment. In the case of the animals fed diet 73, which contained lard heated to 350° for 30 minutes, the initial loss in weight lasted for 4 weeks and amounted to approximately 20 percent of the initial body weight. The animals were able to regain little of this lost weight and appeared to follow a general downward trend in weight for all periods of the experiment,

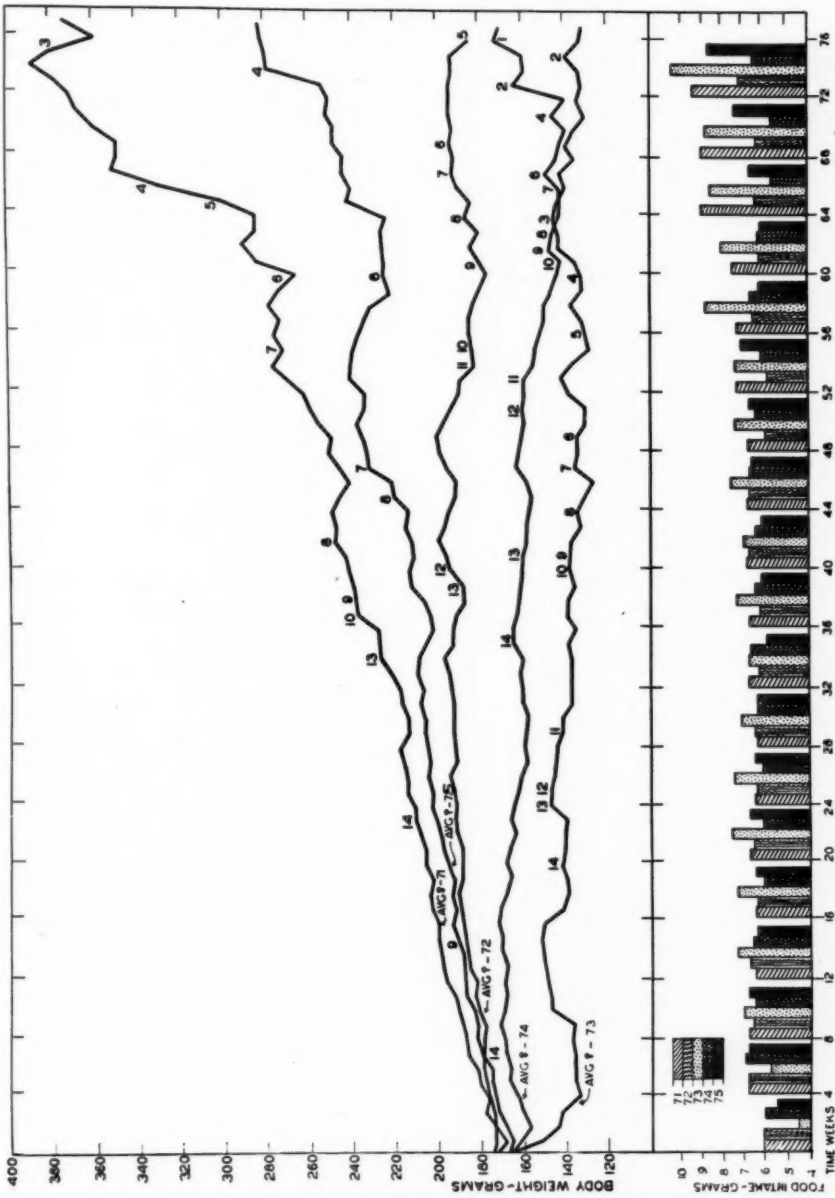

FIGURE 1.—Average body weight and average food intake of female rats fed diets containing 50 percent of heated and unheated lard.

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except between the eighth and fourteenth weeks.

The average body weight of the group fed diet 72, which contained lard that had been heated to 300° for 30 minutes, increased gradually for 6 or 7 months, after which no additional increase in body weight was observed.

In contrast with the three groups that received the heated fat are the two receiving diets 71 and 75, respectively, which contained unheated lard. These groups gained in body weight for the entire experimental period. The group fed the diet containing 12 percent of fatty acids from unheated lard (diet 75) in place of an equal amount of lard were never quite so heavy as the control group that received the diet containing 50 percent of unheated lard.

The food-intake values for the five groups of rats are also given in figure 1. These values are calculated average daily food intakes for periods of 4 weeks per histogram. There appears to be no essential difference in food intake in any of the groups, except the group receiving diet 73 that contained lard heated to 350° C. for 30 minutes. The animals in this group appeared to be more greasy than those in the other groups. It is believed that the calculated greater food intake for this group may be owing to the greater tendency of the animals to smear the food on their paws and bodies. The diet was not only distasteful to the animals but may have been toxic also.

The figures above the body-weight curves (fig. 1) refer to the number of animals surviving at any given time. There appears to be some difference in survival of the rats fed diet 75. In this group 40 percent of the animals were still alive at the end of 75 weeks, while only 20 percent of the controls, 33 percent of the animals on diet 72, 15 percent of those on diet 73, and 7 percent of those on diet 74 were still alive at this time. After about 90 weeks, only 5 percent of the animals were still alive, except in the group fed 12 percent of free fatty acids.

An unusual type of paralysis appeared in a considerable number of the rats fed heated lard. Figure 2 shows a rat in this condition. The animal seemed to be able to lift its head with great difficulty and lay most of the time with head resting on the floor of the cage. It had very poor coordination when walking; instead of lifting its weight on its legs, the animal had a tendency to walk on its hocks, as indicated by the right hind leg (fig. 2). The condition was a progressive type of paralysis in that the first clinical evidence of the condi-

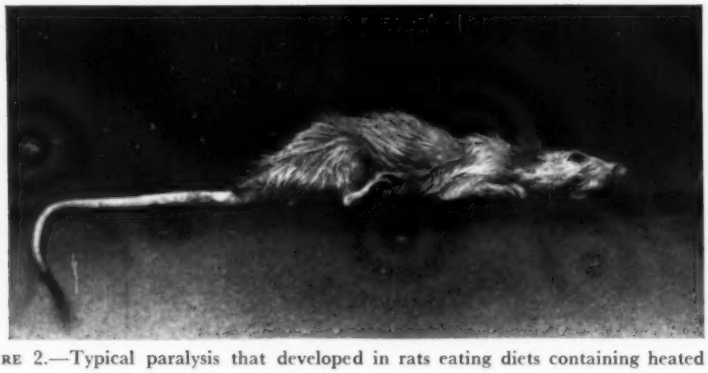

FIGURE 2.—Typical paralysis that developed in rats eating diets containing heated lard.

tion was a pronounced trembling movement of the head, followed by increased inability to use the legs; in some animals paralysis was so pronounced that they could not eat unless the food cup was turned over on the side. The forelegs became involved earlier than the hind legs. This paralysis had some of the characteristics of vitamin E deficiency described by Mackenzie, Mackenzie, and McCollum (*17*). The condition was never observed in rats fed the diets containing either unheated lard or unheated lard plus fatty acids therefrom. It was assumed, therefore, that the paralysis was caused by some factor in the heated fats, which possibly involved destruction of the vitamin E in the diet.

Pathology

One hundred and eleven rats were autopsied. Fifty-five of the animals were permitted to live under the experimental conditions until death and were then autopsied as soon as possible. Later in the course of the experiment, when it became apparent that an animal was about to die, it was killed so that the tissues could be obtained unaffected by autolysis. All tissues were routinely fixed in Zenker's fluid and stained with haematoxylin and eosin. In addition, liver was fixed in formalin (U. S. P. 1:10) for the sudan IV stain and in absolute alcohol for the glycogen stain.

Stomach

Gross examination of the forestomach usually disclosed no apparent lesions. However, in nine of the animals this organ presented varying numbers of discrete, white, nodular elevations of the mucosa measuring up to 3 x 2 mm. Microscopically, such elevations proved to be benign

FIGURE 3.—Forestomach ulcer of rat on diet 73 for 14 months. × 95.

papillomas (fig. 3); apart from these ulcers were sometimes found. They consisted of a defect in the squamous epithelium with nonelevated margins. The base was formed by a richly vascular granulation tissue containing many leukocytes, chiefly eosinophiles. The granulation tissue often extended laterally for a considerable distance between the muscularis mucosae and circular muscular coats.

In some rats, gross examination of the glandular portion of the stomach revealed the presence of large and small discrete ulcers (fig. 4). In some instances as many as five ulcers were present in one stomach. They were usually in the prepyloric region on the lesser curvature. The largest ulcer measured 5 x 3 x 2 mm. The margins were elevated and undermined, and the base was pearly gray. In addition to these well-formed ulcers, irregular hemorrhagic erosions up to 4 mm. in diameter were seen in scattered parts of the pyloric portion of the stomach.

Microscopically, the ulcers appeared as well-defined lesions (fig. 5). In the smaller ulcers, the mucosa of the margins appeared perfectly normal, but in some instances was atrophic and fused to the base of the ulcer (fig. 6). The base of such an ulcer was formed by granulation tissue frequently extending through the muscularis mucosa laterally for some distance beneath the overlying intact epithelium (fig. 7, *A*). There was abundant exudate in this granulation tissue, chiefly polymorphonuclear leukocytes, with many eosinophiles. In some of the ulcers the base contained irregularly fragmented fibers of the muscularis mucosae and many large acini (fig. 7, *B*). The latter occurred in small groups and sometimes suggested the appearance of adenomatous

FIGURE 4.—Large and small pyloric ulcers from rat on diet 73 for 17 months. Normal size.

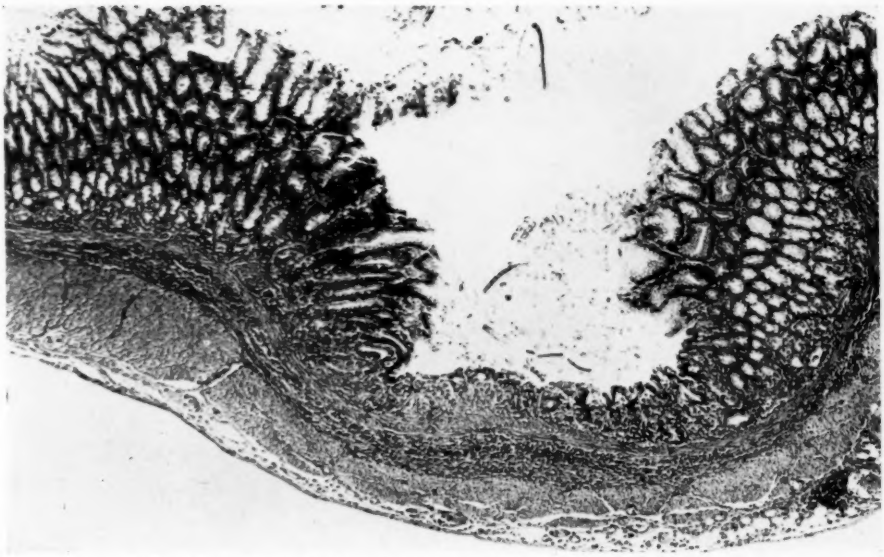

FIGURE 5.—Stomach ulcer of rat on diet 73 for 14 months. × 58.

FIGURE 6.—Margin of stomach ulcer showing atrophic epithelium. Rat was on diet 73 for 14 months. × 85.

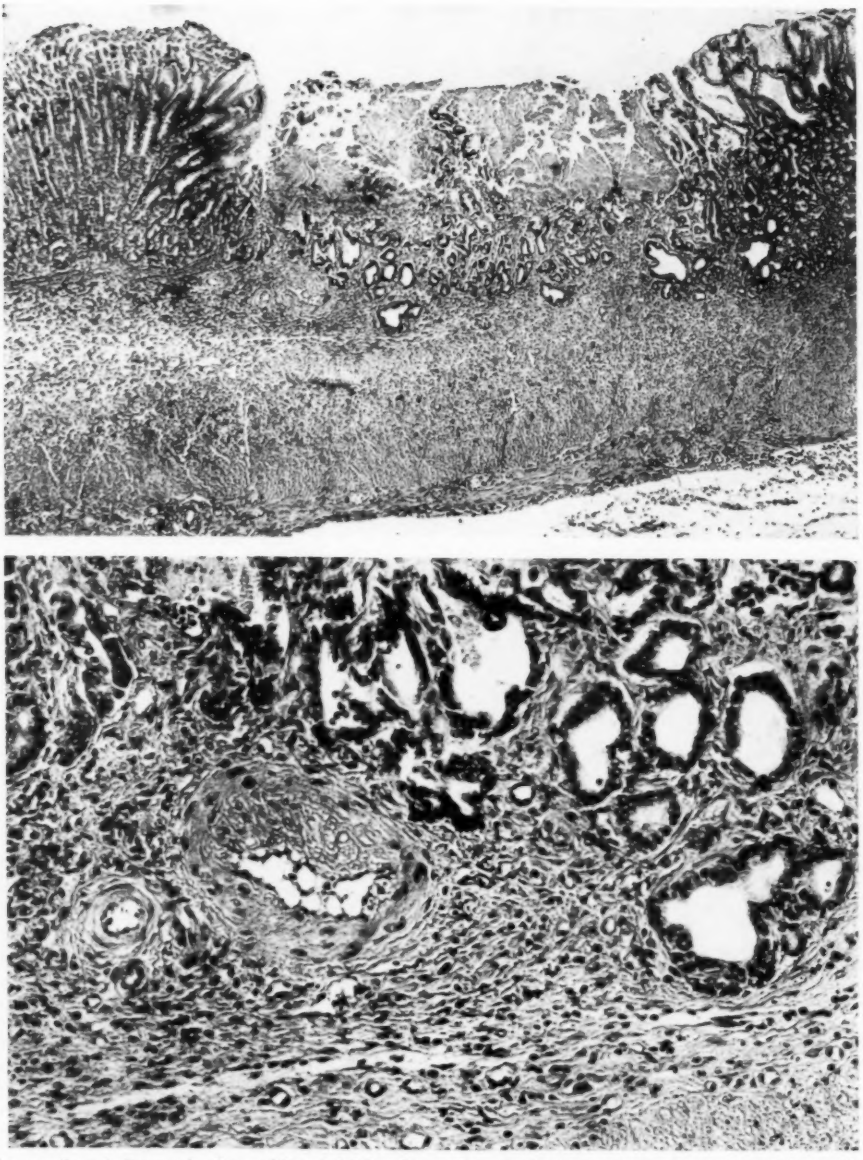

FIGURE 7.—*A*, Stomach ulcer with acini lying beneath muscularis mucosae. Rat was on diet 73 for 17 months. × 42; *B*, Higher magnification of the acini. × 190. (*A*, upper, and *B*, lower part of figure.)

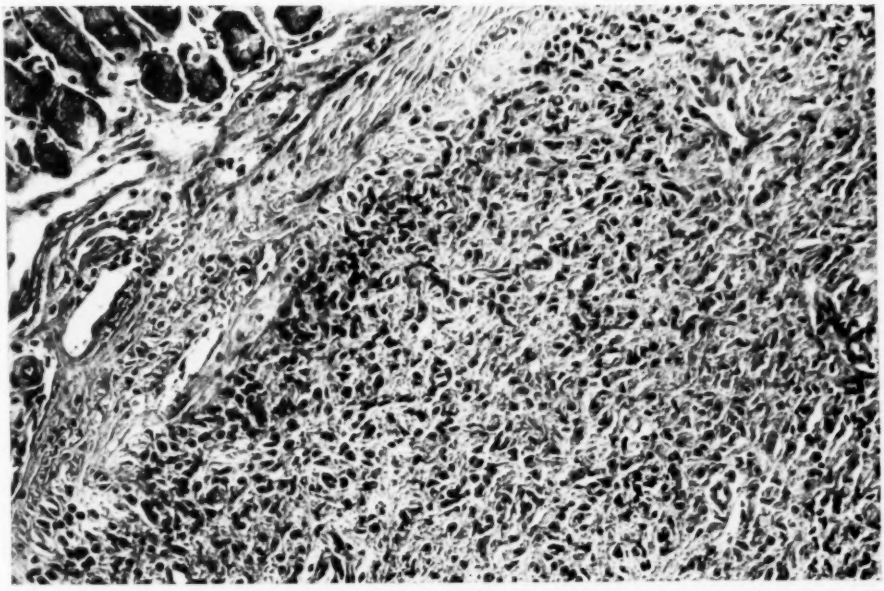

FIGURE 8.—Exuberant granulation tissue in nodulelike formation, from rat on diet 73 for 18 months. × 210.

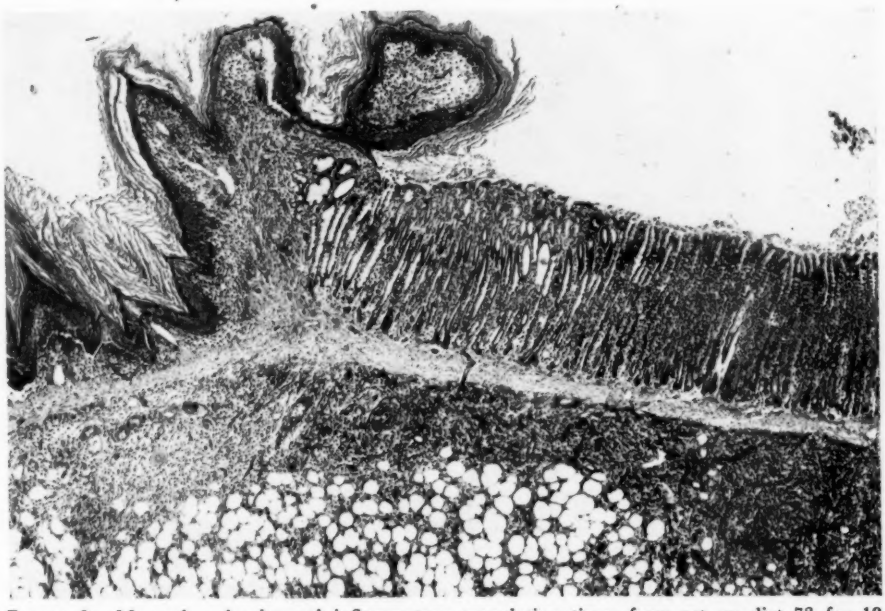

FIGURE 9.—Mucosal and submucal inflammatory granulation tissue from rat on diet 73 for 18 months. × 100.

hyperplasia. However, the acini, though dilated, were not markedly irregular. The individual cells formed single layers in orderly arrangement and were not anaplastic in character. In no instance did these acini penetrate the muscular coats. The acini were surrounded by dense granulation tissue and apparently were associated with regeneration, and an attempt on the part of the ulcer to heal. There was no indication that they represented a carcinomatous process.

Another feature observed grossly was the presence in one animal of several firm, nodular elevations, 4 x 3 x 2 mm., in the pyloric portion of the stomach. The mucosa overlying the nodules was intact in some instances and apparently ulcerated in others. Microscopically, these lesions appeared to be composed of extremely vascular granulation tissue (fig. 8). Because of the character of the lesion, consideration was given to the possibility that it might be a fibrosarcoma or possibly a sclerosing hemangio-endothelioma. However, after careful study with special stains, a diagnosis of granulation tissue was made. One of the confusing features was the occasional penetration of granulation tissue through the muscular coats into the serosa. In view of the fact that transition stages existed between this suggestively infiltrating lesion and other areas of unquestionable granulation tissue, it was felt that a diagnosis of malignancy was not justified. In early lesions the mucosa was edematous and contained great numbers of eosinophiles (fig. 9). Such exudate was also found in the submucosa. In successive stages it was observed that fibrin was present and that subsequently fibroblasts were laid down so that the edematous tissue underwent organization into a vascular fibroblastic granulation tissue. Therefore, these lesions were considered inflammatory processes. Often there was no evident overlying ulceration.

Liver

In the control animals, after about 1 year, the liver appeared enlarged, grossly, and of light red color with smooth contours. Miscroscopically, islands of normal parenchyma were seen, the surrounding tissue showing noticeably fatty changes (fig. 10, *A* and *B*). These changes were characterized in some instances by large fat globules appearing in the cytoplasm and crowding out the nucleus. In other instances, the fat appeared as finely divided droplets in the cytoplasm. Both types occurred in any portion of the lobule. In isolated instances there were focal areas of fibrosis (fig. 10, *C*) in these regions of fatty change, resulting in slight irregularity and retraction of Glisson's capsule. This connective tissue enclosed irregular islands of parenchyma and sometimes radiated from the portal spaces, but other than this had no characteristic distribution. This lesion was considered to be a fatty cirrhosis.

As early as 7 months after the animals were placed on diet 73, gross irregularities were observed in focal areas of the liver. These appeared as gentle, sweeping undulations which later became pitted, granular, and depressed, leaving elevated ridges in the intervening spaces. The liver was not uniformly involved, but at times as much as half of one of the large lobes, particularly at the margins and on the undersurface, showed the lesion. Microscopically, such lesions resembled the picture of acute yellow atrophy (fig. 11, *A* and *B*). The initial event appeared to be focal degeneration of hepatic cells (fig. 12, *A*), occurring in any portion of the lobule or involving several contiguous lobules. The hepatic cells were in various

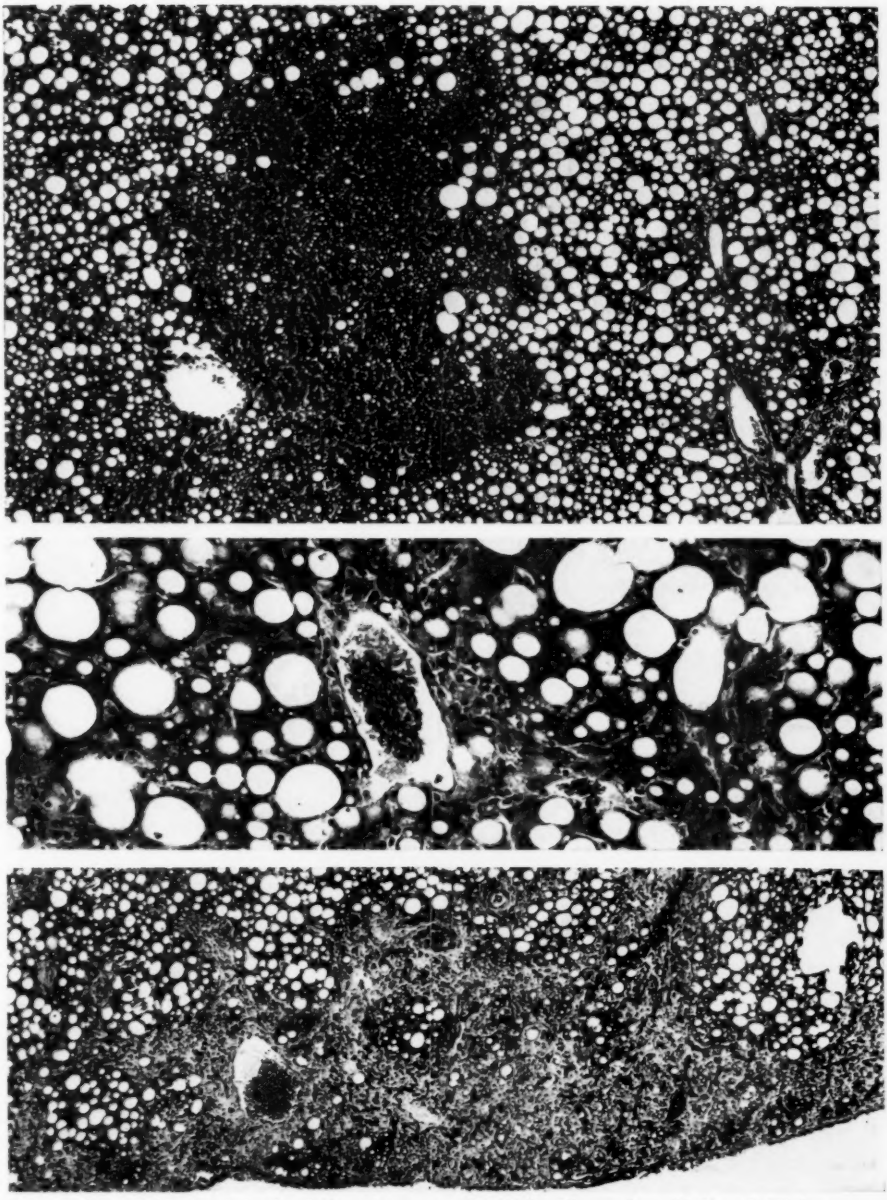

FIGURE 10.—*A*, Fatty cirrhosis of liver in rat on diet 71 for 18 months. × 58. *B*, Higher power than *A*, showing microscopic character of fatty change. × 195; *C*, Fatty cirrhosis and fibrosis of liver in rat on diet 71 for 14 months. × 64. (*A*, top; *B*, middle; and *C*, lowest part of figure.)

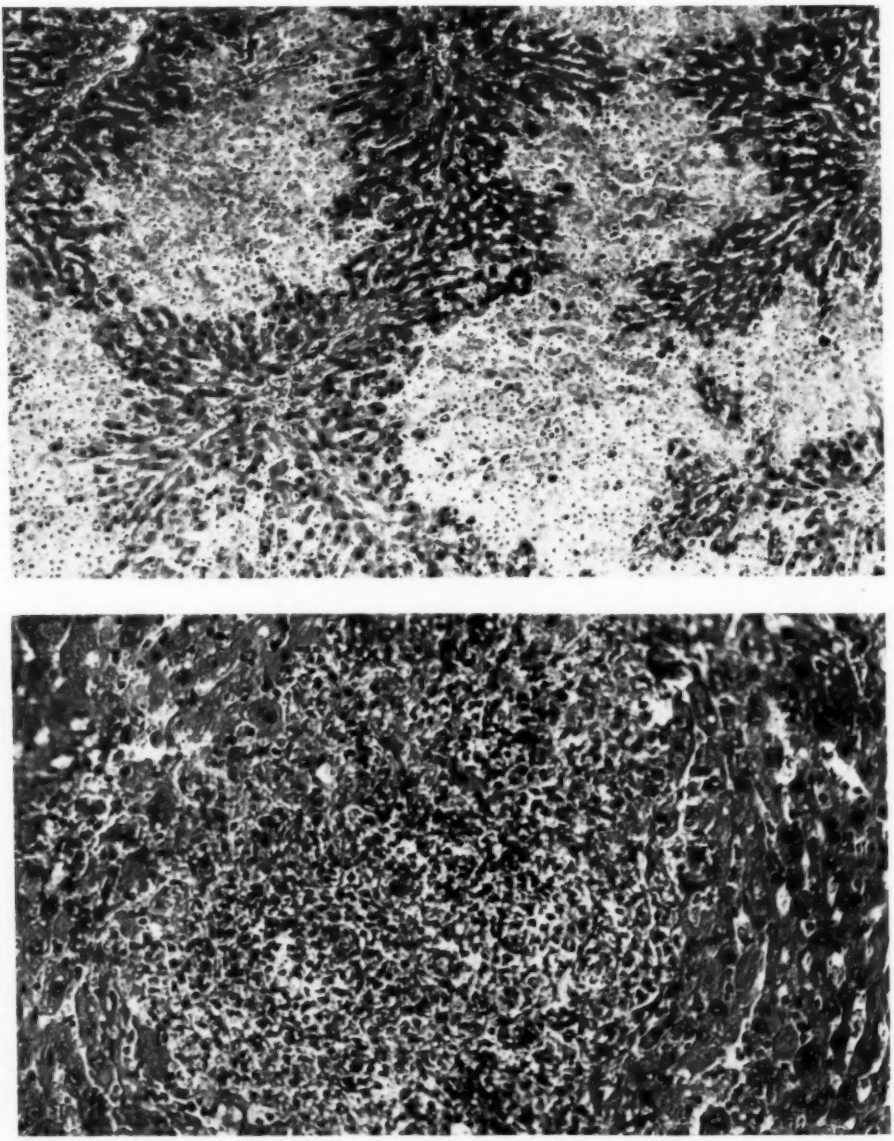

FIGURE 11.—Degenerative hepatitis. *A*, From rat on diet 73 for 9 months. × 95. *B*, From rat on diet 73 for 17 months. × 265. (*A*, upper, and *B*, lower part of figure.)

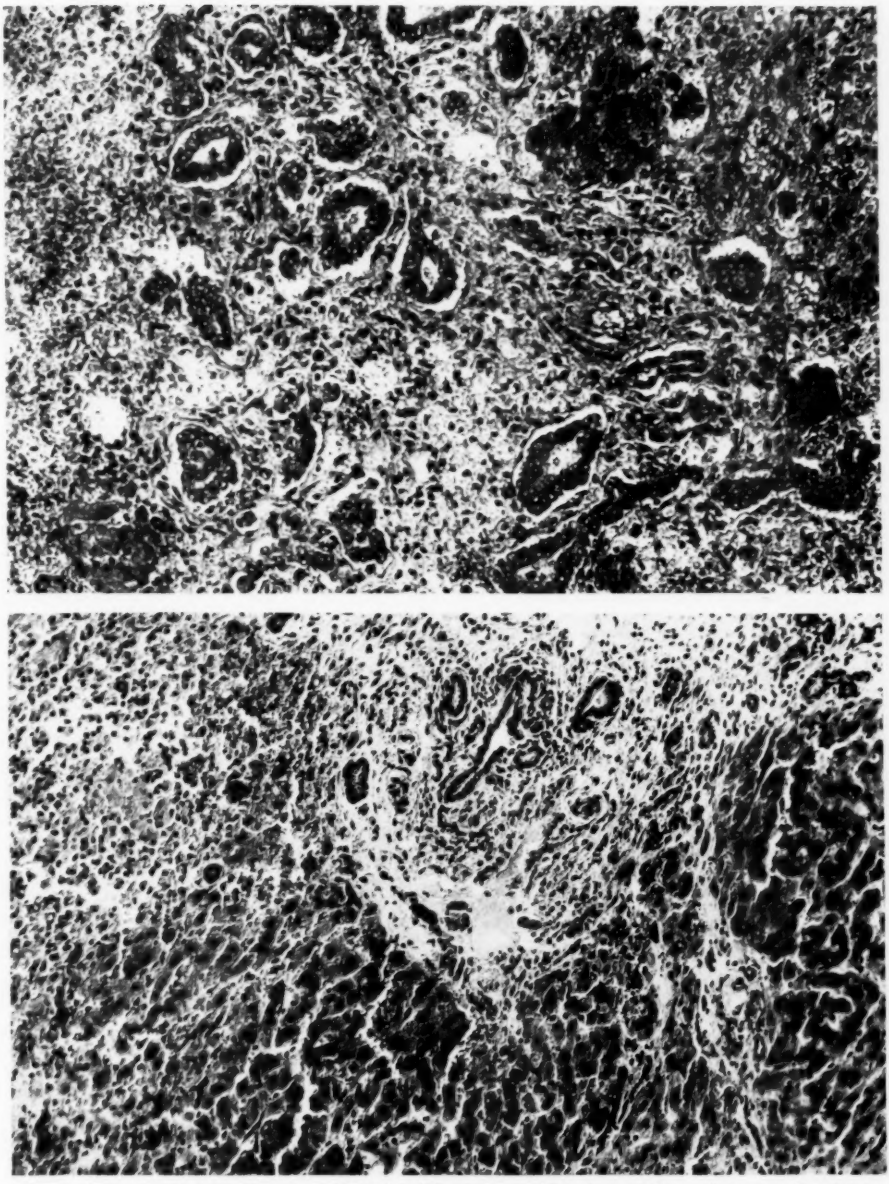

FIGURE 12.—*A*, Degenerative hepatitis with bile-duct proliferation of liver of rat on diet 73 for 7 months. × 235. *B*, Degenerative and proliferative hepatitis of liver of rat on diet 73 for 12 months. × 155. (*A*, upper, and *B*, lower part of figure.)

stages of degeneration with the pyknotic nuclei sometimes representing the only remaining cellular debris. These necrotic areas infrequently became infiltrated with polymorphonuclear leukocytes, but rarely, if ever, with any lymphocytes or plasma cells. This degenerative process frequently involved the parenchymal cells around the portal spaces, leaving the bile ducts still well-preserved. When there was sufficient collapse of parenchyma, many bile ducts appeared crowded into a small area, and sometimes there was epithelial hyperplasia with three or four layers of cells (fig. 12, *B*). Scattered through such areas was a slight proliferation of fibroblasts, sparsely strung out between bile ducts and remaining hepatic cells. This lesion was considered a degenerative hepatitis, eventuating into cirrhosis in some cases.

Heart

Grossly, no lesions were observed in the myocardium, but microscopically, there were many focal areas of myocardial degeneration and fibrosis (fig. 13, *A*). These areas appeared not unlike healing infarcts. The lesions were not related to a disturbance in the coronary vessels, however, since the latter appeared intact. Muscle fibers, individually and in groups, became necrotic and disappeared, followed by fibrosis. Inflammatory cells were not present. This lesion was considered to be focal degenerative myocardial fibrosis.

Kidney

Grossly, the capsule of the kidney usually appeared smooth, but occasionally there were small retracted areas. In such areas minute cortical scars were present microscopically (fig. 13, *B*), which may not have been the result of experimental procedure, but rather the aging of the animals. These lesions were also observed in old control animals, but less frequently. In the experimental animals, the lumina of the collecting tubules were frequently filled with granular debris. The exact nature of this material was not determined. It did not contain iron or red blood cells. Often the lining cells were atrophic and showed areas containing mitotic figures. No exudate was found between adjacent tubules. This lesion appeared to be of the nature of a tubular nephrosis.

Table 2 gives the histologic changes which were observed in the liver and glandular stomach of rats on these heated fat diets. Most of the degenerative hepatitis occurred in the group fed the diet heated to the highest temperature. No degenerative hepatitis was found in the control group, and only one animal had hepatitis in the group receiving diet 75,

TABLE 2.—*Liver and stomach lesions in rats fed heated lard diets*

Diet No.	Heat treatment of lard		Liver			Glandular stomach		
	Temperature	Time	Fatty changes		Degenerative hepatitis cases	Ulcer	Carcinoma	Sarcoma
			Moderate	Marked				
	°C.	*Minutes*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*
71	None (control) [1]		10	8	0	1	0	0
72	300	30	8	3	5	2	0	0
74	300	120	7	2	1	1	0	0
73	350	30	12	0	13	4	0	0
75	None; 12 percent unheated fatty acids [2]		9	2	1	0	0	0

[1] 1 animal had a highly anaplastic sarcoma of the lung at 26 months.
[2] 1 animal had a lung neoplasm, a bronchiogenic carcinoma, when 27 months old; 1 ovarian tumor and also myocardial degeneration and fibrosis were present in an animal 27 months old.

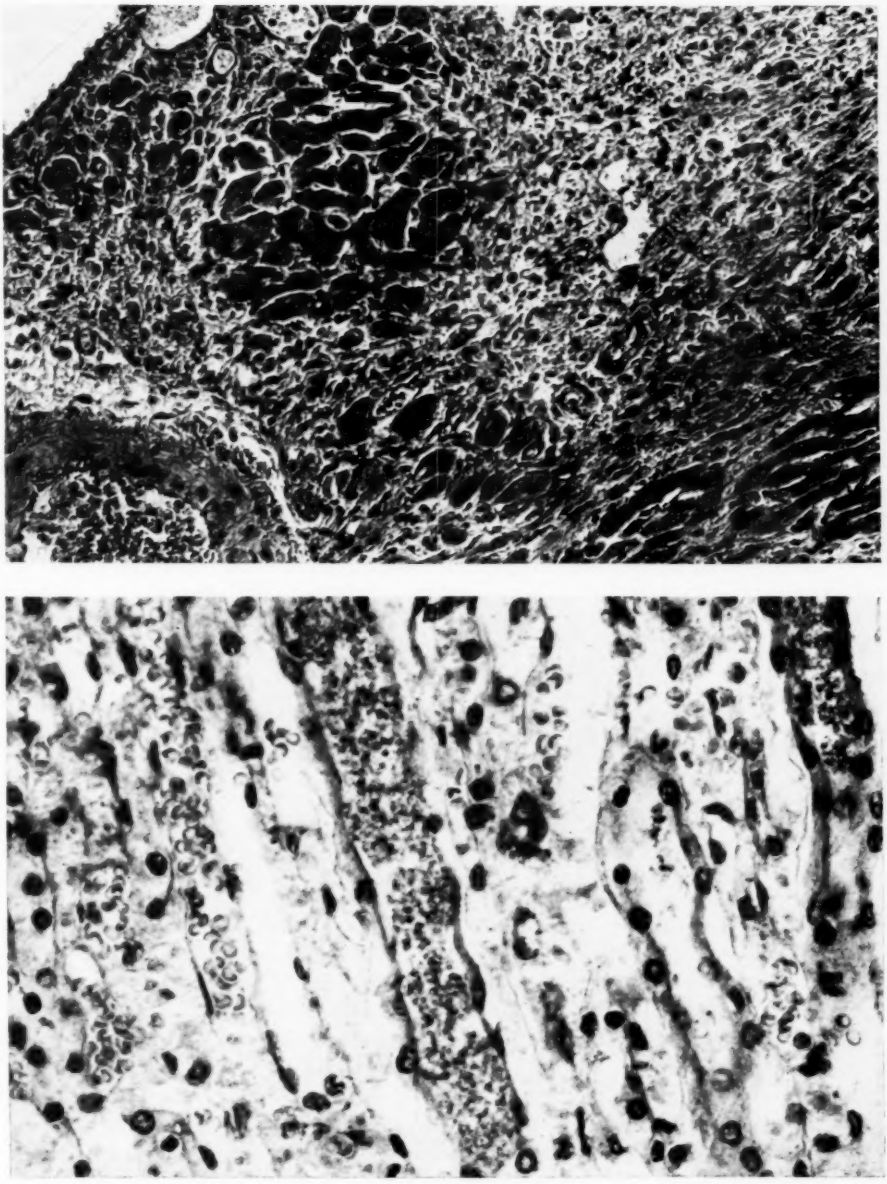

FIGURE 13.—*A*, Myocardial degeneration and fibrosis in heart of rat on diet 72 for 18 months. × 225; *B*, Nephrosis in rat on diet 74 for 15 months. × 415. (*A*, upper, and *B*, lower part of figure.)

which contained unheated lard and free fatty acids from unheated lard. Half of the glandular ulcers observed were in the animals that received the diet heated to the highest temperature. One ovarian tumor and two lung neoplasms were found in rats 26 to 27 months old. These lesions were probably of spontaneous origin.

DISCUSSION

It is impossible to say at this time why the experiments described herein did not give results similar to those obtained by Roffo. There are few statistics available on stomach and liver lesions normally present in old rats. Roffo's protocols indicate that some animals lived longer than those in the present experiment. However, only about one-half of the total number of rats with which Roffo started are accounted for in his published records.

The diet Roffo used appears to have been less rich in several essential dietary constituents, as compared with the basal diet used in this work. Therefore, it is impossible to say whether or not basal-diet differences could have been responsible for the striking difference in the lesions found in the stomach and other organs. There were many animals in our groups receiving heated fat which showed very definite clinical evidence of toxicity. The chemical decomposition of heated lard observed by Larsen and Morris (*18*), which appeared to be a function of the time and temperature of heating, resulted in the disruption of some double bonds. Triglycerides were rapidly disrupted above 330° C. We believe the resulting fatty acids, or their radicals, combined in part in the form of unsaponifiable aliphatic ketones. Digitonin-precipitable cholesterol disappeared in the lard heated at 350° for 30 minutes. This decomposition of the lard components by heat seems likely to have produced toxic materials. The histologic evidence of toxicity observed in the liver, heart, and kidney of many animals in the groups fed heated fat may have been due to some of these as yet unknown pyrolytic products.

Experiments have now been started to determine the type of lesion normally present in the stomachs of old rats on a stock diet. It is of interest to determine whether or not the chronic deep ulcers observed may be of frequent occurrence in old rats.

SUMMARY

Five groups of rats were fed five nutritionally adequate diets containing 50 percent of lard, for 27 months. In two diets the lard was heated to 300° C. for periods of 30 minutes and 2 hours, respectively; in another, the lard was heated to 350° for 30 minutes. The lard in the other two diets was not heated.

There was very little difference in survival between the control group and the groups receiving heated fat, at 21 months, when only about 5 percent of the original animals were still alive. In the group of rats fed 12 percent of the fatty acids of unheated lard in place of an equal amount of lard, 25 percent were still alive at 21 months.

A progressive type of paralysis having some characteristics of vitamin E deficiency in adult rats was observed in some of the animals that were fed the heated-fat diets. The paralysis first involved the forelegs and head and was accompanied by tremors; in the later stages a definite lack of co-ordination in walking was observed.

Rats maintained on a diet consisting of 50 percent of unheated lards were able to increase in body weight during the experimental period. Other rats, maintained on diets containing heated lard in the same amount, were scarcely able to maintain their body weight, and many gradually lost

weight during the entire experimental period.

Neither gastric nor hepatic neoplasms were found under the experimental conditions employed. Chronic gastric ulcers appeared in a few rats, and cirrhosis of the liver in many fed the heated-fat diets.

Lesions observed in the heart and kidney are also described.

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Influence of Irradiation-Killed Cells on Tumor Growth

By PAUL S. HENSHAW, *senior radiobiologist,* with the technical assistance of HENRY L. MEYER, *scientific aide, National Cancer Institute, National Institute of Health, United States Public Health Service*

Clinical experience has shown repeatedly that tumors can be made to regress and in some cases disappear completely by treatment with roentgen and gamma rays. Just how such radiations act to cause tumor regression, however, is not fully understood.

From a wide variety of experimental evidence, especially that dealing with free living cells, it is known that cells may be killed by the direct action of radiation. Although it seems certain that direct killing action is responsible in part for tumor regression following irradiation (*1*), there is evidence that indirect action also must play a part. Changes produced outside or even remote from the cells in question appear to have an influence, as illustrated by the following facts: (1) Tumor cells irradiated in vitro are more resistant than when irradiated in vivo (*2, 3*); (2) tumor cells transplanted to an irradiated area are less likely to grow than when transplanted to a nonirradiated area (*4*); and (3) irradiated tumor cells that are undergoing satisfactory regression will grow (and kill the host) if transplanted to a new location (*5, 6*).

Three possible types of indirect action may be listed: (1) Damage to the blood vascular system resulting in an altered tumor metabolism; (2) modification of both the stroma and malignant cells followed by leukocytic infiltration, fibrosis, and encapsulation; and (3) generation of toxic tissue products (necrotoxins). It is the purpose of this report to present experimental results dealing with the last of these.

It is at least plausible that the decomposition products of cells killed outright by the radiation may exert a detrimental effect on remaining viable tumor cells. Three types of experiments have been carried out to investigate the influence of irradiation-killed tumor cells on the behavior of viable tumor cells.

In all cases, mouse sarcoma 37 was used in young Dilute Brown male mice. Ordinarily, 0.02 to 0.04 ml. of finely macerated pulp of this tumor inoculated subcutaneously will produce tumors in 100 percent of the animals. The tumors grow rapidly and kill the host animal in 10 to 20 days. Roentgen doses of 5,000 r applied to such tumor pulp before inoculation prevents all tumor growth. Furthermore, similar doses applied tangentially to well-established tumors growing in vivo cause complete regression in most (85–90 percent) of the cases. These results form the basis of the present study; and by way of indicating reliability, it may be pointed out that such findings have been obtained almost routinely in a long series of tumor-irradiation experiments involving such steps.

EXPERIMENTAL PROCEDURE

EXPERIMENT 1

An adequate amount of select tumor material was obtained under aseptic conditions, put through a small hand press for maceration, and placed in two small

circular aluminum boxes with covers. The latter provided proper aseptic conditions and served as convenient vessels for holding the material during treatment.

X-rays were obtained under the following conditions: 200 kv., 20 ma., 25 cm. distance and 0.25 mm. copper plus 0.55 mm. aluminum filter. The intensity at the point of treatment was 200 r per minute, making the exposure for 5,000-r doses 25 minutes.

Following treatment, both treated and nontreated pulp materials were drawn up into separate syringes and carefully measured into aluminum containers in such a manner as to obtain mixtures of the following proportions: 25 percent irradiated pulp plus 75 percent nonirradiated, 50 percent irradiated pulp plus 50 percent nonirradiated, 75 percent irradiated pulp plus 25 percent nonirradiated. Ten inoculations were then made with each type of mixture, and the host animals were observed for tumor growth.

Tumors were obtained in all cases; the tumors grew rapidly and killed the animals in 10 to 20 days. It is clear, therefore, that an excess of nonviable irradiation-killed cells in the injection masses did not inhibit tumor formation.

Experiment 2

In the second experiment 10 well-established subcutaneous tumors, 4 to 5 mm. in diameter, were obtained. Irradiation-killed pulp was then secured, as described previously, and was inoculated subcutaneously into the region immediately surrounding the growing tumor masses. Five or six blebs of the nonviable tumor pulp were injected around the growing tumor in such a manner as to surround the growing tumor with dead tumor pulp 3 to 4 times its size. The lengths and breadths of the growing tumors were measured at regular intervals, and the times of death of the host animals were recorded.

Again it was found that the rate of growth was not impaired by the presence of nonviable tumor material and that death of the host animal occurred as normally in 10 to 20 days.

Experiment 3

As mentioned, 5,000 r applied tangentially to the tumor causes complete regression in 85 to 95 percent of the cases. The host animal's body was shielded with lead in such a manner that only the tumor mass was exposed to the direct beam of the rays. Tumors exposed to doses of 5,000 r in this manner are usually so severely damaged that the tissues slough leaving an ulcer that heals slowly.

Ten animals were selected which bore tumors 10 to 15 mm. in diameter, and the tumors were exposed to 5,000 r as described. Immediately following treatment, 0.04 mg. of normal viable pulp was injected directly into the irradiated tumor masses. Thus, effectively, a small mass of viable tumor was again enveloped in a much larger mass of dead tumor material, in this case including an intact vascular and connective tissue bed.

Noting the behavior of the tumors, we found that sloughing occurred as usual and that slow-healing ulcers appeared. However, without exception new tumors arose in the scarred areas. Hence, since only a small percentage of recurrences were found in control experiments where no injections were made, it seems clear that at least part of the new growths arose from the inoculations. Furthermore, since all new tumors grew as rapidly as controls after they were once established, it is obvious that the viable tumor cells experienced no ill effects from the presence of irradiation-damaged tumor tissues in the immediate vicinity.

SUMMARY AND CONCLUSION

Three types of experiments have been carried out to investigate the influence of irradiation-killed tumor cells on other living tumor cells.

In the first case, killed and viable pulp were mixed in varying proportions before subcutaneous inoculation; in the second, well-established small tumors were enveloped in subcutaneously inoculated irradiation-killed pulp; and in the third, viable cells were inoculated into the center of the large irradiation-killed tumors. In all cases, the viable cells grew as though no dead cells were present in the immediate neighborhood.

It may be concluded, therefore, that so far as the material tested is concerned, irradiation-killed cells or their decomposition products exert no inhibitory influence on the growth potentiality of viable tumor cells.

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Induction of Pulmonary Tumors in Mice With Ethyl Carbamate (Urethane)

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Some months ago the writers noted with surprise that the lungs of 26 of 29 young C3H female mice in a certain experimental series showed multiple (1 to 6) pulmonary tumors. These animals had received only local roentgen-ray exposure to a skin fold drawn out from the animal body with surgical thread. Grossly and microscopically, these tumors resembled in every respect the pulmonary tumors of mice described by other investigators (*1–12*). Since the normal incidence of such tumors in this strain of mice was known to be less than 5 percent, the treatment procedure was examined to determine if possible the factors responsible for tumor induction.

Although the bodies of the animals had been protected from the direct beam of the radiation, it was known that some scattered radiation penetrated to all parts. Moreover, anesthesia (urethane) was administered by intraperitoneal injection to facilitate handling. Thus, three possible carcinogenic agents could be listed: (1) Direct radiation, which produced changes in the skin and which affected the lungs secondarily; (2) scattered radiation, which acted directly on the lungs; and (3) the anesthetic.

TEST FOR CARCINOGENIC AGENT

Experiments were devised to test each of the factors individually and in all possible combinations, using the same age, strain, and sex of mice, and the same experimental treatment. The treatments consisted of 14 weekly intraperitoneal injections, with an interval of 7½ months between the beginning of treatment and autopsy. The dosage of urethane was 1 ml. of a 10-percent aqueous solution per 100 gm. of body weight, or 0.25 ml. per mouse, as the animals weighed uniformly 23 to 26 gm. at the time they were treated. Since in previous experiments the tumors appeared independently of the size of the dose of radiation applied, an intermediate dose of 500 r only was used. The results are shown in table 1.

With the exception of 1 animal in series 5, lung tumors occurred only in those

TABLE 1.—*Lung tumors produced with various treatments*

Series No.	Treatment	Animals at beginning	Animals surviving treatment	Animals showing lung tumors	
				Gross examination	Microscopic examination
		Number	*Number*	*Number*	*Number*
1	Anesthetic only	10	9	8	7
2	Anesthetic and direct rays	10	9	7	7
3	Anesthetic and indirect rays	10	9	8	8
4	Anesthetic, direct and indirect rays	20	18	16	16
5	Direct rays only	10	9	1	1
6	Indirect rays only [1]	10	10	0	0
7	Controls	10	9	0	0
8	25 r whole body [2]	10	9	0	0

[1] The combination of direct and indirect rays without anesthesia is not shown. This combination was started, however, but anesthesia was given twice by mistake early in the treatment series. Five of nine animals showed tumors.

[2] It was estimated on the basis of ionization measurements that for doses of 500 r applied to skin folds as described, doses of about 25 r of scattered radiation reached the lungs. As another experimental variation, this dose was administered directly to the whole body.

animals to which anesthetic had been given. Thirty-eight of the fifty animals receiving urethane showed tumors, whereas only 1 of the 40 that did not receive urethane showed a single tumor.

Urethane is a comparatively simple compound ($NH_2COOC_2H_5$) and is one of the simplest chemical agents found thus far to have carcinogenic effects. Numerous questions were raised by the new findings, and new avenues of approach were opened. What was the minimal number of treatments that would induce tumors? What was the minimal dosage of urethane and the minimal time of appearance? Was it possible that this simple agent acted directly on the cells of the lung alveoli to cause malignant changes? Were the tumors caused by repeated anesthesia or by impurities in the urethane? Did the anesthesia serve to release a latent virus? An approach to some of these problems lay through the detailed study of the lungs and tumors of experimental animals and through additional experiments.

Pathology of the Tumors and Associated Lesions

Grossly, the lung tumors observed in C3H mice, as well as those in strain A mice were found chiefly to lie scattered beneath the pleura (fig. 1), although some were seen to be deeply situated in the lung parenchyma. The nodules measured up to 2 or 3 mm. in diameter, were semitransparent and often slightly elevated. Occasionally we confused a minute subpleural focus of lymphoid cells with tumor, but invariably these were listed as questionable at the time of gross examination. All doubtful cases were examined microscopically, every tenth serial section being studied. While the peripheral index is usually considered as an adequate quantitative measure of the occurrence of these tumors, the figures obtained are only approximate in any case, since some tumors do not extend to the surface. Aside from noting the gross similarity of these tumors to those previously detailed in the literature, our description will be confined chiefly to the microscopic aspects, the early phases of tumor formation, and the associated lesions.

The lungs were routinely fixed in Zenker's formol solution for microscopic examination. Paraffin sections were made, and the tissues were stained with haematoxylin and eosin in the usual manner.

The earliest lesions which could be identified with certainty as neoplastic appeared to be of two types. One consisted of an accumulation of glandlike cells in a region which may or may not be connected with a terminal bronchus. By the examination of multiple sections, it was sometimes possible to observe an intimate association of early tumor with a bronchus. Figure 2, *A,* shows a small, but nevertheless well-established tumor consisting mainly of glandlike cells. Figure 2, *B,* shows a group of enlarged hyperchromatic cells along the walls of an alveolus which may be an earlier stage of this type of tumor. The neoplastic character of these cells, together with their similarity in appearance to well-established tumors of the same type (figs. 2 and 4), warrants calling the cell groups adenomas. The other type of tumor occurred much less frequently. It was composed of large, pink-staining cells with dark nuclei and had no particular pattern (fig. 3). Whether these tumors should be considered apart from the adenomatous type is as yet not certain, although the distinctions may be traced back to very early stages. The origin of both tumor types is not clear.

Both types as a rule show few or no inflammatory cells and almost no mitosis. When inflammatory cells do occur, they

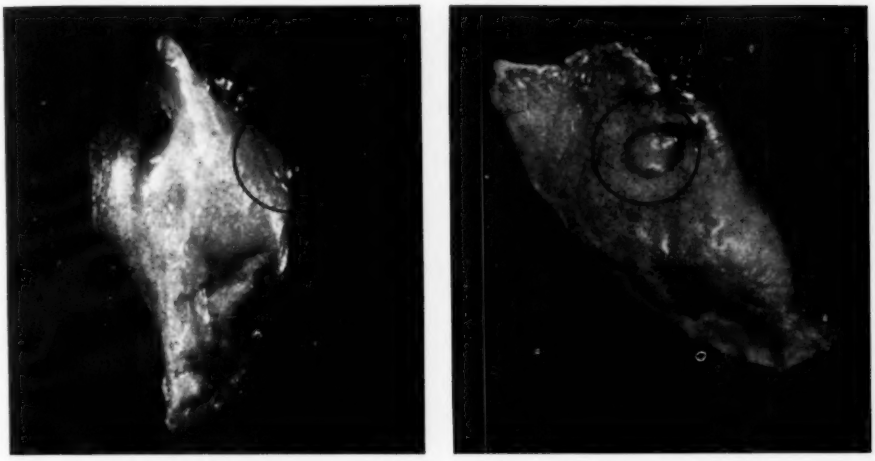

FIGURE 1.—Gross appearance of tumor nodules in the lungs of C3H mice.

are of the lymphoid-cell type and are found in groups (figs. 2, 3, and 4).

It is interesting that the different tumors appear to arise in the absence of significant damage to lung tissues. Microscopic examinations of the lungs of both control and tumor-bearing animals showed moderate emphysema and pleural thickening. The same was true for peribronchial lymphoid cell accumulations. Some cases of bronchiectasis, purulent bronchitis, and, rarely, acute pneumonia were seen, but they were distributed alike among experimental and control animals.

As early as 8 to 12 weeks after treatment started, curious small cellular foci were found (fig. 5). While the significance of these lesions is not clear, it is suspected that the type shown in figure 5, *A*, is the early lesion associated with the massive, monocytic, lipoid-type pneumonia due to occur later, and that shown in figure 5, *B*, while not greatly different from *A*, is more likely to be precedent to the lung tumors (figs. 2, *A*, and 4, *A*, and *B*). The focus in figure 5, *A*, is composed of large and small monocytic cells of a single variety (interspersed with lymphocytes), whereas in figure 5, *B*, there are two varieties of cells, one clearly monocytic and similar to the large ones in *A*, and the other elongated, with scant cytoplasm and containing an elongated dark staining nucleus. Whatever the nature of these foci, they gradually disappeared as the experiment progressed. In the later states, either true lung tumors or massive, monocytic pneumonia could be seen (fig. 6). It seems significant that lung tumor and pneumonia were never seen in the same animal. The lungs showed either tumor or pneumonia, but never were the two found associated.

Since the pneumonia-type lesions seen here may occupy whole lobes or large segments thereof and since they are made up essentially of monocytic-type cells, with scattered lymphocytes and few neutrophiles, the lesions are suggestive of the virus-type pneumonias (*13*, *14*). The question has therefore been raised whether urethane may not act through a virus-type mechanism in producing pulmonary tumor. This possibility is mentioned here only because we are not in a position at this time to pursue virus investigations.

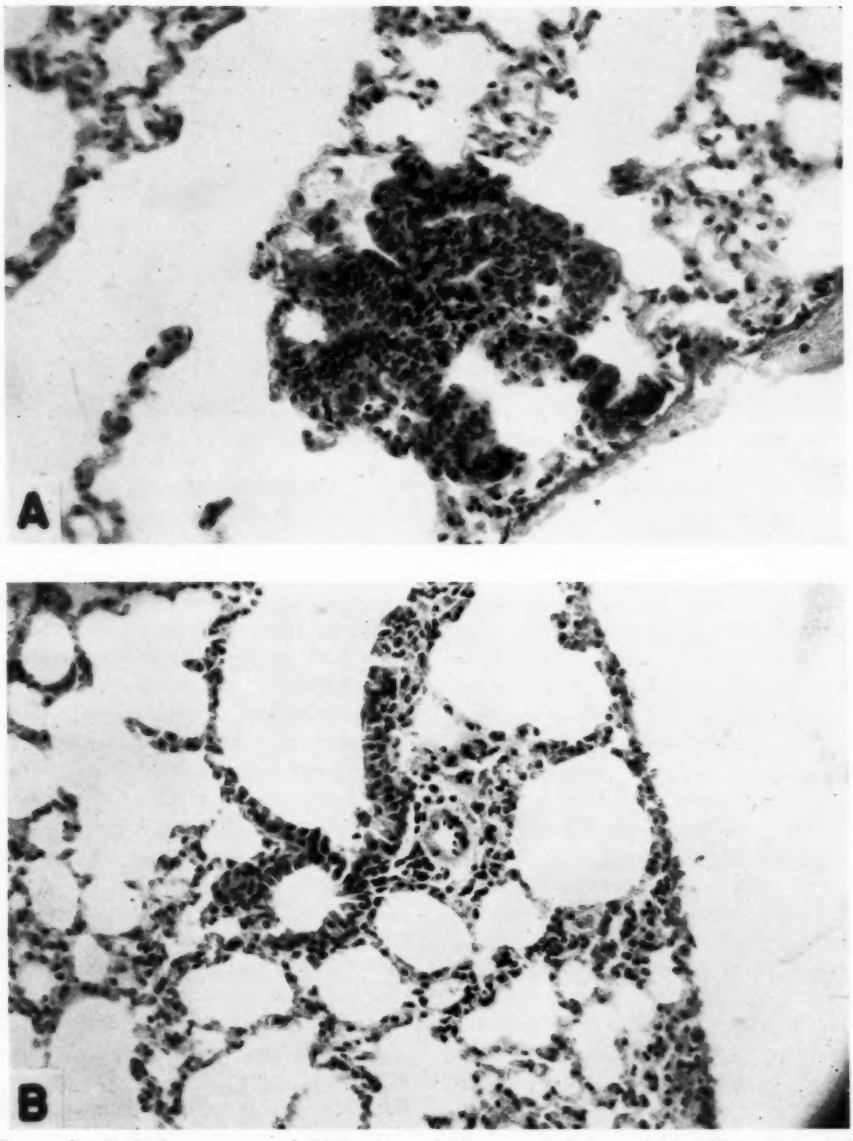

FIGURE 2.—Early lung tumors of C3H mice. *A*, An accumulation of glandlike cells possibly associated with a terminal bronchus; *B*, Hyperchromatic, and enlarged cells along the wall of an alveolus. Haematoxylin and eosin stain. × 200.

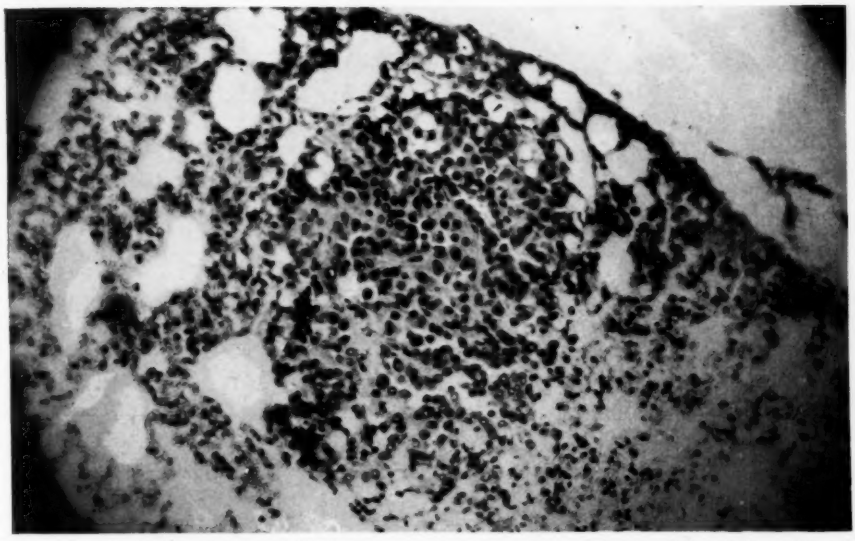

FIGURE 3.—Early lung tumor of C3H mice, composed mainly of large pink-staining cells with dark nuclei and having no particular pattern. Haematoxylin and eosin stain. × 200.

MINIMAL NUMBER OF TREATMENTS

An experiment was carried out to determine the smallest number of anesthetic treatments required to produce lung tumors. Six lots of mice of the same strain, age, and sex as described previously were used. Minimal anesthetizing doses injected intraperitoneally were given in every case. Autopsies were performed within 5 months after the treatment series started. The results are shown in table 2.

In this experiment, one animal of the control group showed a single lung nodule (the only nontreated animal of all the experiments to show a lung tumor). The treated animals show a graded incidence of tumors with respect to dosage. While the results suggest that one or two treatments may be sufficient to induce tumors, the figures are not large enough to be statistically significant. The idea that only one or two treatments may produce tumors, however, is strengthened by the evidence in footnote 1, table 1. In this case two anesthetizing treatments were given and five out of nine animals developed tumors. It is possible that more tumors would have been seen in the present series if an interval of more than 5 months had been allowed before autopsy. The results are sufficient, however, to show that the tumors were induced by the application of a small number of anesthetizing doses.

TABLE 2.—*Lung tumors appearing after different number of treatments*

Series No.	Weekly treatments	Animals at beginning	Animals at end	Animals with lung tumors
	Number	*Number*	*Number*	*Number*
1	0	10	9	1
2	1	10	10	2
3	2	10	10	2
4	4	10	10	3
5	8	10	7	3
6	16	10	9	7

MINIMAL TIME OF OCCURRENCE

An experiment involving 60 animals was set up and carried out in the same man-

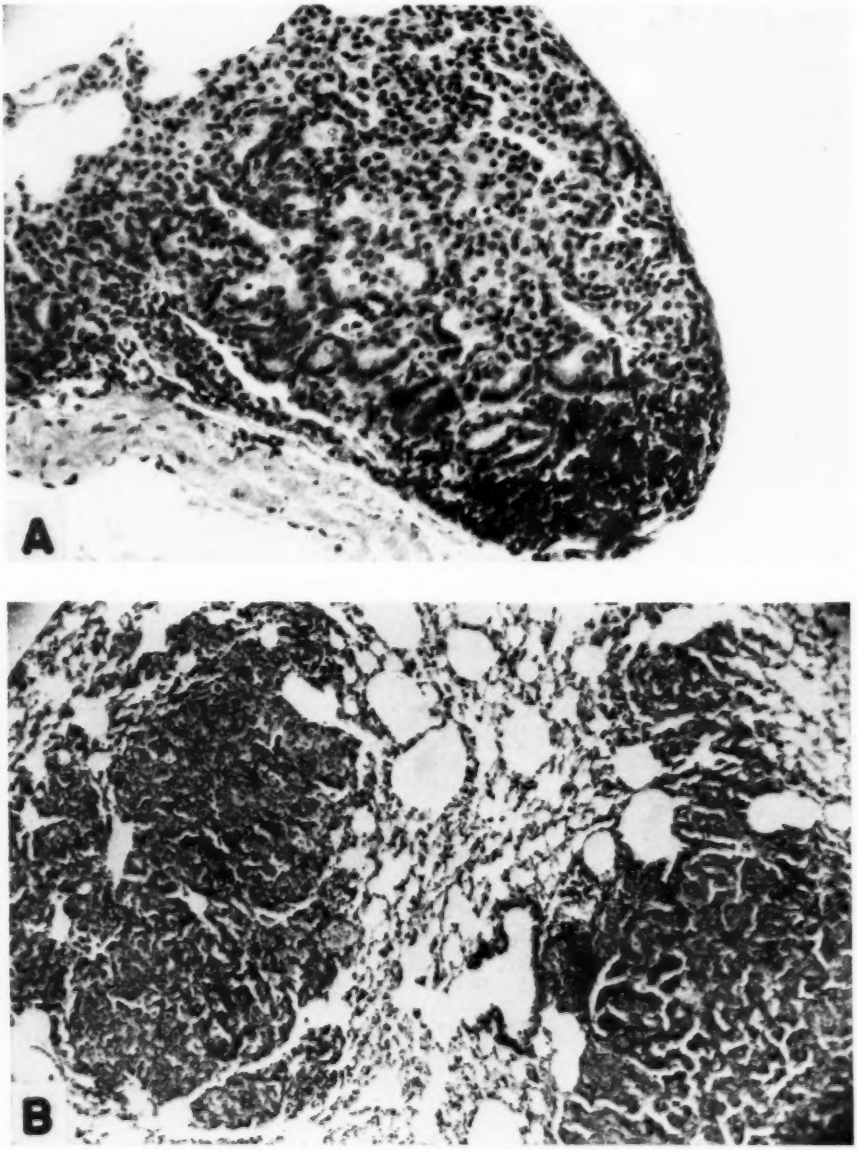

FIGURE 4.—Lung tumors. *A*, Well-established tumor of a C3H mouse. × 200; *B*, Closely associated tumors in a strain A mouse. × 7. Note the isolated lymphoid accumulations in both *A* and *B*. Haematoxylin and eosin stain.

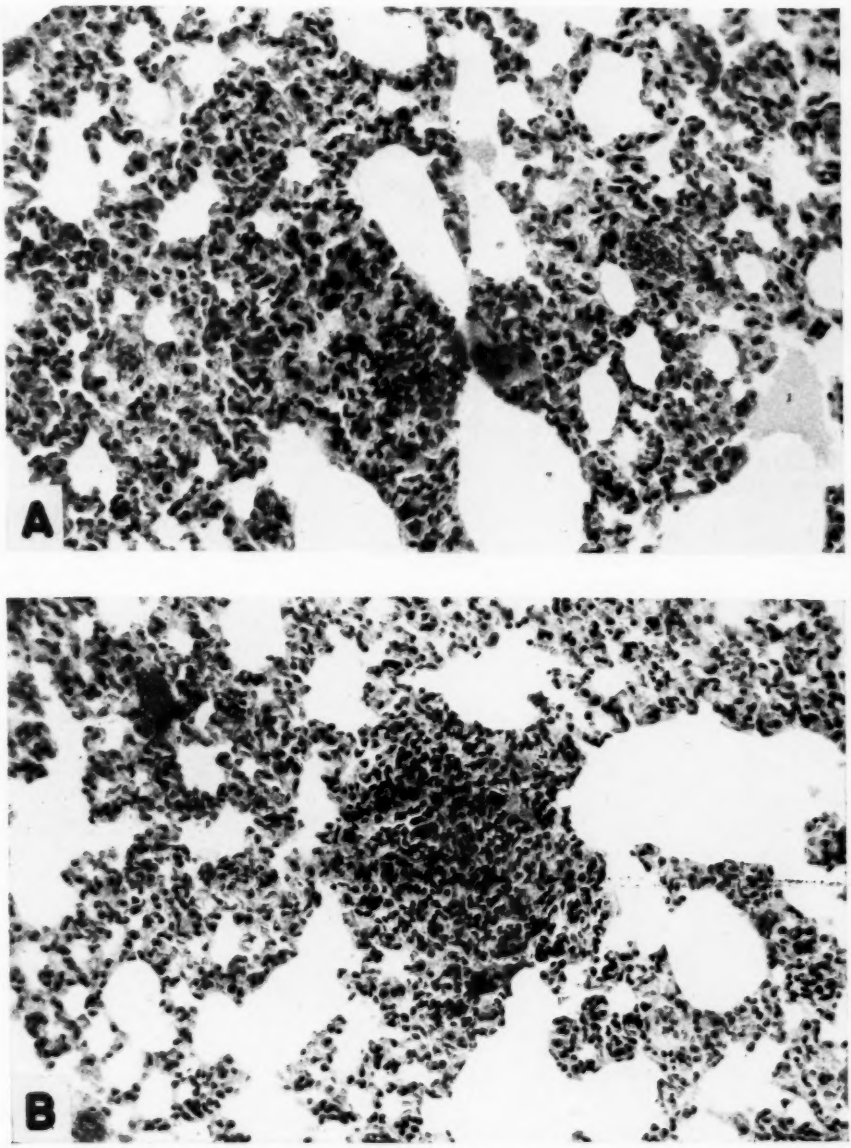

FIGURE 5.—Cellular accumulations in treated animals. *A*, Lesion possibly associated with early pneumonia; *B*, Possible early lung tumor. Haematoxylin and eosin stain. × 200.

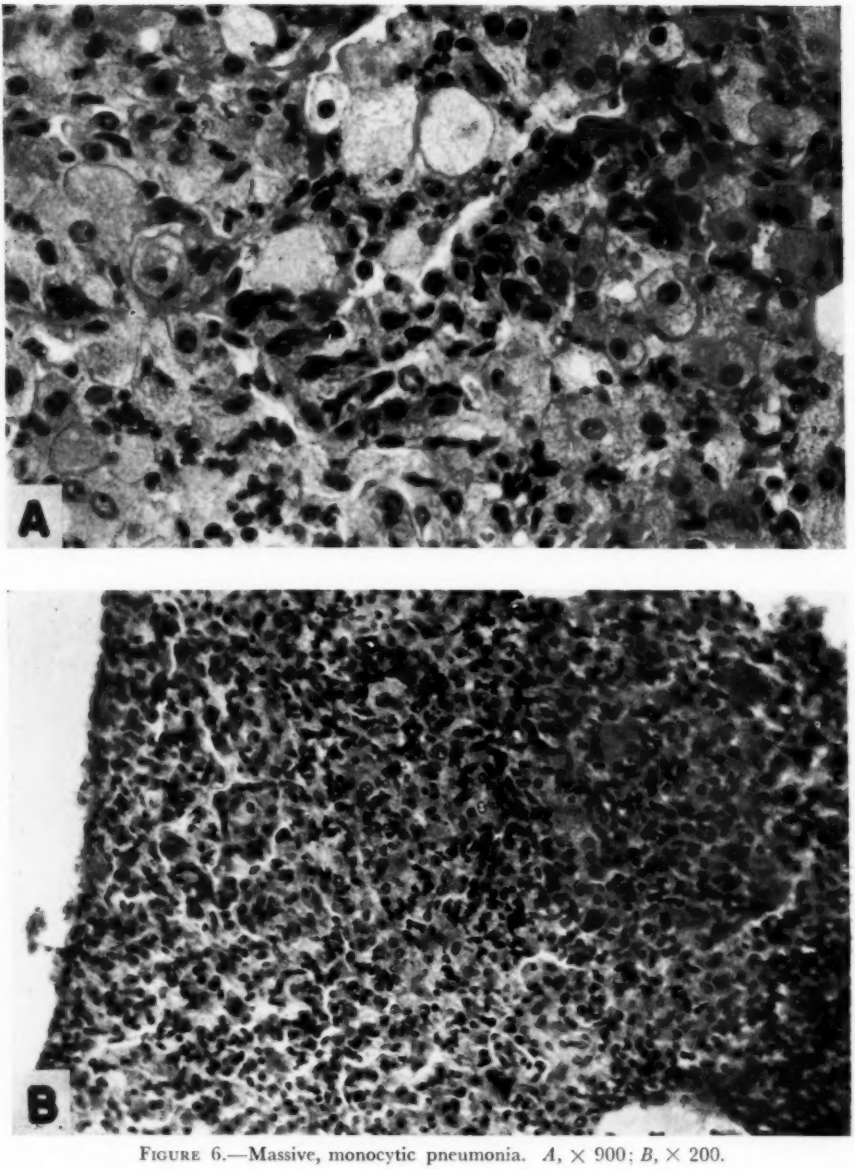

FIGURE 6.—Massive, monocytic pneumonia. *A*, × 900; *B*, × 200.

ner as that for determining the minimal number of treatments, except that 2 animals from each series were killed each month after treatment started and the lungs were examined for tumors. The results are shown in table 3.

As indicated, three criteria may be utilized for determining the presence of lung tumors: The presence of tumors seen grossly, the presence of tumors seen microscopically, and the presence of cellular foci seen microscopically. Since it was found that gross observations of tumors were usually confirmed by microscopic observations, these divisions may in a sense be considered to represent different stages of development of the tumor, the cellular foci representing the earliest morphologic evidence of tumor formation, the microscopic tumor representing an established tumor but one not visible to the unaided eye, and the gross tumor an established visible tumor.

Table 3 shows that at 2 months some evidence of tumors was seen in those animals that received four or more weekly treatments. Likewise, at 3 and 4 months, similar evidence was seen when fewer treatments were given. These findings are consistent with the work of Grady and Stewart (*11*), who found evidence of lung tumors as early as 6 to 8 weeks after injection of 1, 2, 5, 6-dibenzanthracene, and suggest that the rate of malignant change in lung tissue is probably the same irrespective of the nature of the inciting agent.

Effect of Urethane on Lung Tumor Incidence in Strain A Mice

It has been shown by Heston (*10*), Bittner (*9*), and others that the normal incidence of lung tumors in strain A mice [1] is above 75 percent in animals 18 months of age. Ten strain A mice were given 14 weekly treatments of urethane as described, and 10 others were kept as controls. All animals were killed 4½ months after the treatments started. All 10 control animals survived the treatment interval and 1 lung tumor was found in 1 animal at this time. Only 8 of the experimental animals survived treatment, but all showed numerous (45 to 64) peripheral tumors (fig. 7), which were morphologically the same as those described. The results indicate that the time of occurrence of lung tumors due to arise spontaneously later in life was moved forward.

[1] *Albino mice inbred for the spontaneous occurrence of lung tumors.*

Table 3.—*Time of occurrence of tumors* [1]

Number of treatments	Tumor development at different times			
	2 months	3 months	4 months	5 months
0 (controls)				
1			Microscopic tumor; gross tumor.	
2		Microscopic tumor	Microscopic tumor	
4	Single focus; microscopic tumor.	Multiple foci; microscopic tumor.	Microscopic tumor, gross tumor.	Microscopic tumor; gross tumor.
8	Multiple foci; microscopic tumor.	Multiple foci; microscopic tumor.	Gross tumor	Gross tumor.
16	Multiple foci; microscopic tumor.	Multiple foci; microscopic tumor.	Microscopic tumor; gross tumor.	Microscopic tumor; gross tumor.

[1] The various notations indicate in a general way the degree of tumor development at different times. "Single focus" means that a single focus of cells was seen microscopically in the sections examined; "multiple foci" refers to the fact that more than one such focus was seen; "microscopic tumor" indicates that a small, but well-established tumor was seen microscopically, but none was seen grossly; "gross tumor" means that one or more well-established tumors were seen grossly.

FIGURE 7.—Lungs from strain A mice. Upper row: lungs from nontreated controls killed at 7 to 9 months of age. Lower row: lungs from animals receiving 14 weekly treatments with urethane and killed at the same age.

SUBANESTHETIZING DOSES OF URETHANE

It was important to determine if possible whether the anesthetizing action of the urethane was responsible for the malignant changes observed. Ten C3H animals were given the same amount of urethane (0.25 ml., 10 percent-aqueous solution) each week for 14 weeks but in subanesthetizing doses, 0.07 ml. being injected on Monday, Wednesday, and Friday of each week. Thus the same amount of urethane was introduced into the animals each week but not in sufficient amounts at any time to produce anesthesia. Eight of the ten animals survived 4½ months at which time all were killed. Four of the eight showed one to three well-defined tumors.

It appears that anesthetic action is not an essential part of the induction process, thereby necessitating that attention be directed toward other effects of urethane.

DIFFERENT LOTS OF URETHANE

The question arose whether induction was brought about by the urethane (which was chemically pure) or by some impurity. Urethane from two different lots (packed by different distributing houses) and a purified sample were tested.

Purification was accomplished by crystallization from a mixture of ether and hexane. The fact that the melting point was not raised by more than 0.1° C. shows that organic impurities were present in very small amounts. However, ether-insoluble impurities as well as volatile substances responsible for the odor of the CP.

material were removed. The final product was practically odorless and melted at 48.5°–49.5° C. (corrected).

Positive results were obtained with all three lots of urethane in more than half of the animals tested. The findings therefore do not support the idea that the inductions were due to impurities.

SUMMARY

Intraperitoneal injections of minimal anesthetizing doses of ethyl urethane repeated at weekly intervals was found to increase the incidence of lung tumors in C3H female mice from less than 5 percent to more than 75. Two lots of urethane (CP.) as well as a purified sample, were tested and positive results obtained.

The tumor type was found to be largely the characteristic lung adenoma, although a possible second type with different features was seen. Both appeared to arise in a tissue which showed little or no injury.

Preliminary results indicate that the tumors may be detected within 2 to 3 months after treatments start and that only a few treatments (perhaps only one or two) are required to bring about their induction.

Subanesthetizing doses of urethane increased lung tumor incidence significantly.

In animals having a high incidence of spontaneous lung tumors (strain A mice), the time of occurrence of the tumors was moved forward several months by the injection of urethane.

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Ascorbic Acid Content of Tumors and Homologous Normal Tissues

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The ascorbic acid (vitamin C) concentration in several neoplasms has been reported by previous investigators (*1–4*). However, with the exception of Fujiwara et al. (*5*) and Iki (*6*), who determined ascorbic acid in normal liver and hepatic tumor, and Carruthers and Suntzeff (*7*), who determined the vitamin in epidermal carcinoma and epidermis, none of the workers has compared the concentration in tumors with that in the normal tissue of origin. The necessity of comparing biochemical characteristics of neoplastic tissues with those of suitable normal control tissues in order to elucidate changes which occur owing to malignancy, is obvious. This paper presents the results of the determination of ascorbic acid in tumors, in the normal tissues in which the tumors arose, and in tissues of homologous histologic type. Although the question of which cell of the normal tissue is the cell of origin has not been answered for all of the tumors studied, we believe that the comparison between tumor and tissue of origin will aid in the differential characterization of normal and malignant tissues.

METHODS

The tumors analyzed were, with the exception of the primary spontaneous mammary adenocarcinomas, transplanted tumors of the rat and mouse carried at this Institute. Comparable normal tissues were taken from the same strain of animals, unless otherwise noted. Since rats and mice are able to synthesize ascorbic acid and presumably maintain a physiologic concentration in tissues, no specific dietary control was used and the animals were allowed to eat an ascorbic acid free diet, Purina dog chow, ad libitum.

The animals were killed by decapitation or cervical dislocation; the tissues were removed immediately, then freed as far as possible of necrotic material and of encysted or superficial fluid and blood. Ascorbic acid was determined in 5 percent metaphosphoric extracts of these tissues by titration with 2,6-dichlorphenolindophenol by using the method of Bessey and King (*8*). Duplicate analyses were run when possible on individual tissues, but for some of the smaller organs it was necessary to pool corresponding tissues from several animals approximately as follows: lymph nodes from 3 animals, pyloric stomach from 5 and forestomach from 10 mice, small intestine from 2 mice, and lungs and salivary glands from 4 mice. Previous investigators have shown that the concentration of ascorbic acid in various tumors determined by titration with dye agrees with that determined by bioassay (*9, 10*) and by spectrophotometric measurements (*10, 11*). The results of this study are predicated on the assumption that this finding could be extended to all tumors.

RESULTS

Hepatoma 31 in Osborne-Mendel rats (*12*), and spontaneous hepatomas 587 (*13*) and 98/15 (*14*) in strains A and C3H mice, respectively, were especially favorable tumors for comparison with normal tissues of origin. Not only has their origin

TABLE 1.—*Ascorbic acid in hepatoma and liver*

Tissue	Number of animals	Ascorbic acid per 100 gm. of fresh tissue	
		Range	Average
		Milligrams	*Milligrams*
Hepatoma 31 in rats	9	23–40	32
Normal rat liver	13	15–29	21
Regenerating rat liver [1]	8	13–22	18
Fetal rat liver [2]	7	15–25	20
Hepatoma 587 in A mice	6	23–36	30
Hepatoma 98/15 in C3H mice	6	18–26	22
Normal mouse liver [3]	35	21–41	32

[1] 72 hours after partial hepatectomy.
[2] Livers of fetuses from each of 7 mothers pooled. The stage of pregnancy ranged from the seventeenth to the twentieth day.
[3] The values for strains A, C3H, dba, and C57 Brown mice were averaged as no strain difference was evident.

from hepatic parenchymal cells been established, but these parenchymal cells comprise over 90 percent of normal liver. Furthermore, two rapidly growing homologous tissues, fetal and regenerating liver, were available for comparison. The ascorbic acid concentration in hepatic tissues is presented in table 1.

The concentration of ascorbic acid in hepatoma 31 averaged 32 mg. per 100 gm. of fresh tumor which was about 50 percent higher than in normal liver. This was about the concentration found by Fujiwara et al. (*5*) in the transplanted Ikubo hepatoma but was considerably higher than that found by Iki (*6*) in primary hepatomas induced by feeding rats *p*-dimethylaminoazobenzene. No significant differences were observed between fetal and regenerating liver and normal liver even though the first two tissues grow at an accelerated rate. Actually the average concentration in regenerating liver was somewhat lower than that in normal liver. It was, therefore, apparent that the increased growth rate of hepatoma, which, however, was less than that of fetal or regenerating liver, could not be directly correlated with the high vitamin concentration.

The ascorbic acid concentration in the mouse hepatomas was slightly lower than in rat hepatoma 31, although their tissue of origin, mouse liver, actually contained more ascorbic acid than did rat liver. Hepatoma 98/15, a very slow-growing tumor, contained less ascorbic acid than hepatoma 587, and the possibility suggested itself that within one tumor type ascorbic acid and rate of growth might be correlated. This question will be discussed in more detail later.

The ascorbic acid concentration of various lymphomas and lymph nodes was determined (table 2). Histologic evidence indicated that the lymphomas arose in lymph nodes, but the exact cell of origin has not been established. However, the bulk of the cells in a lymph node are lymphoidal elements, and the node was considered a suitable homologous tissue for comparison with these tumors.

The mouse leukosis 72942 [1] and the Murphy rat lymphosarcoma (*15*), both localized lymphomas which do not produce a leukemia until late in their development, contained lower concentrations of ascorbic acid than did the lymph nodes in which

TABLE 2.—*Ascorbic acid in lymphomas and normal lymph nodes*

Tissue	Number of animals	Ascorbic acid per 100 gm. of fresh tissue	
		Range	Average
		Milligrams	*Milligrams*
Murphy lymphosarcoma in rats	7	23–36	29
Leukemic nodes and thymus of rats injected intraperitoneally with Murphy lymphosarcoma	6	27–43	33
Normal rat lymph nodes	12	42–51	48
Leukosis 72942 in A mice	23	26–58	38
Leukosis P1534 leukemic nodes in dba mice	21	40–56	47
Leukosis Y103 leukemic nodes in dba mice	6	66–75	70
Normal mouse lymph nodes	12	45–48	46

[1] *Leukosis 72942 arose spontaneously in a male strain A mouse. The primary tumor was diagnosed as a leukemic lymphomatosis, and although certain variations appeared in later generations of the transplant, Dr. G. B. Mider considered this diagnosis as probably correct.*

they arose. In contrast therewith, the ascorbic acid concentration of the affected nodes of mice with leukosis Y103,[2] a generalized leukemia, was about 50 percent higher than that of normal nodes; in fact, this was the highest concentration found in any of the neoplasms studied or previously reported. The leukemic nodes of mice with leukosis P1534,[3] a generalized leukemia similar to Y103, contained the same amount of ascorbic acid as did nodes from normal mice. These generalized leukoses also involved liver and spleen, but these tissues were not found to contain appreciably more ascorbic acid than comparable normal tissues (34 mg. per 100 gm. and 43 mg. per 100 gm., respectively) although they were heavily infiltrated with lymphocytes.

When transplanted intraperitoneally, the Murphy lymphosarcoma produced a generalized leukemia which killed the rats within a week. The concentration of ascorbic acid in leukemic nodes and thymus of these animals was about the same as in the local tumor which arose upon subcutaneous injection.

An especially large variation, which correlated with the age of the tumor, was observed in the ascorbic acid concentration of leukosis 72942. The concentration in the oldest tumors was approximately twice that in the youngest. This correlation is shown in figure 1. None of the other neoplasms examined showed a similar relation between tumor age and ascorbic acid content. The possibility was considered that this increase in ascorbic acid might be related to an acceleration of rate of growth.

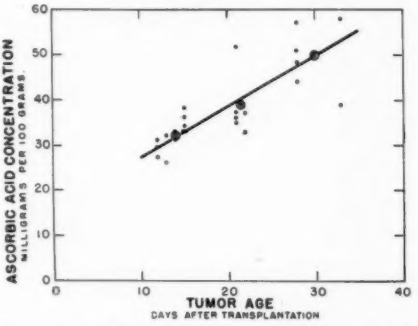

FIGURE 1.—Effect of tumor age on the ascorbic acid content of leukosis 72942.

The average growth rate, based upon increase in weight of tumor, was, however, constant from the twelfth to the twentieth day, and no correlation could be established.

The ascorbic acid concentration in several tumors of glandular origin and in their respective normal tissues is shown in table 3. In each case, except the adrenal cortex, the normal organ is a complex of different types of tissue and would not be a suitable comparison. Thus, the gastrointestinal system comprised smooth muscle as well as glandular tissue, whereas the hyperplastic mammary tissue and lungs contained large amounts of connective as well as glandular tissue. It was possible to control this factor in studies of intestine and glandular stomach by making use of a larger animal and separating muscle and mucosa. The result of the determination of ascorbic acid in the separated muscle and mucosa of the rabbit showed unexpectedly that the concentration in stomach mucosa and smooth muscle was identical, and that the concentration in intestinal smooth muscle and mucosa was also the same but considerably higher than in the stomach. If one assumes that distribution of ascorbic acid is similar in the mouse, then the concentration in the whole small intestine or whole gastric portion of

[2] *Leukosis Y103 arose in a strain dba (212) mouse after painting with methylcholanthrene. Dr. G. B. Mider considered the cell type involved in this generalized leukosis as probably being a very immature lymphoid element.*

[3] *Leukosis line P1534, a spontaneous "stem-cell" leukemia, was obtained from Law (16) and has been transplanted with uniform success in mice of the dba strain, subline 212.*

TABLE 3.—*Ascorbic acid in tumors of glandular origin and in normal glandular tissues*

Tissue [1]	Number of animals	Ascorbic acid per 100 gm. of fresh tissue	
		Range	Average
		Milligrams	*Milligrams*
Adenocarcinoma of the pyloric stomach in C3H mice (17)	4	19–24	21
Pyloric stomach of C57 Brown mice	22	21–22	22
Mucosa of rabbit stomach	2	6–7	7
Adenocarcinoma of the small intestine in A mice (18)	5	41–46	43
Small intestine of fasted A mice [2]	11	52–61	55
Intestinal mucosa of rabbits	2	27–40	34
Spontaneous mammary adenocarcinoma [3]	35	19–57	34
Hyperplastic mammary tissue of lactating C3H mice	4	10–11	10
Hyperplastic mammary tissue of pregnant C mice	4	8–10	9
Adenoma (adenocarcinoma?) (20) of the adrenal cortex in strain C mice	8	16–23	20
Adrenal cortex of cattle [4]			150
Pulmonary adenocarcinoma F in A mice (22)	7	31–36	33
Lungs of mice [5]	24	18–28	24

[1] Numbers in parentheses refer to literature references in which the tumors are described.

[2] Hopkins (19) reported a twofold increase in rat intestinal ascorbic acid due to fasting. The concentration in mice feeding normally would probably be somewhat lower than the values reported here.

[3] Tumors from C3H, C, C×C3H, and I×C3H mice. No strain differences were observed.

[4] Data for cattle adrenals reported by Glick and Biskind (21). Several investigators have found similar concentrations in the adrenals of other species.

[5] The values for strains A, C, and C3H mice were averaged as no strain differences were observed.

the stomach might justifiably be compared with that in the corresponding tumor.

The ascorbic acid concentrations in tumors which do not fit into the classifications in the preceding tables are given in table 4. The tumors analyzed were salivary-gland tumors in two strains of mice (13), a rhabdomyosarcoma (23), a squamous-cell carcinoma of the forestomach (24), an osteogenic sarcoma (25), an osteoma (26), and four fibrosarcomas, Jensen sarcoma, Crocker sarcoma 180, S37, and fibrosarcoma 4801 (27). For all tumors the ascorbic acid concentration of a comparable normal tissue was determined.

Reported analyses of ascorbic acid concentrations in the tissues of normal animals indicate a progressive diminution with age

TABLE 4.—*Ascorbic acid concentration in tumors and homologous tissues*

Tissue	Number of animals	Ascorbic acid per 100 gm. of fresh tissue	
		Range	Average
		Milligrams	*Milligrams*
Spindle-cell carcinoma of salivary gland in A mice	5	14–17	15
Spindle-cell carcinoma of salivary gland in C mice	6	17–24	20
Retrolingual salivary gland [1]	47	19–49	33
Rhabdomyosarcoma in C mice	6	19–30	26
Leg muscle	6	3.7–4.9	4.3
Squamous-cell carcinoma of the forestomach in C57 Brown mice	6	15–26	19
Forestomach	29	34–41	38
Jensen sarcoma in rats	5	30–49	39
Fibrosarcoma 4801 in C3H mice	3	21–25	23
Crocker sarcoma 180 in C mice	39	9–31	19
S37 in dba mice	2	18–21	20
Connective tissue fascia from rabbits	2	1.7–3.6	2.7
Omentum (fat-free) from rabbits	2	1.9–5.5	3.7
Osteogenic sarcoma (Barrett) in C3H mice	4	35–40	37
Fetal bone from swine	4	6.5–7.8	7.0
Bone tumor 118/85 in I × C3H mice	5	20–23	21

[1] The values for salivary glands from strains A and C mice were averaged.

(8, 28). The effect of the age of the host on the concentration of ascorbic acid in tumors was investigated. The results appear in table 5. The age of the host appeared to exert no significant influence on the ascorbic acid concentration of the three neoplasms studied. Possibly the use of very large samples might increase the significance of the small difference found

TABLE 5.—*Effect of age of host on ascorbic acid concentration in tumors*

Tumor	Age of host	Tumors analyzed	Ascorbic acid concentration	
			Range	Average
	Months	*Number*	*Milligrams*	*Milligrams*
Crocker sarcoma 180 [1]	2	6	15–20	18
Do	24	6	17–23	19
Leukosis 1534	ca. 2.5	5	43–52	46
Do	> 14	6	40–49	44
Spontaneous mammary adenocarcinoma	5.5–8.0	8	26–45	33
Do	24.0–28.7	9	19–57	29

[1] Crocker sarcoma 180 in 2-month-old mice reached an average size of 2.6 gm. after 12 days, whereas the tumor in 24-month-old mice weighed 1.2 gm. after 13 days.

in the case of spontaneous mammary adenocarcinomas.

Crocker sarcoma 180 was transplanted into various tissues to examine the effect of site on the ascorbic acid content. The results (table 6) show no differences, with the possible exception of the slightly elevated values found for the tumors which grew intradermally. However, this difference might be owing to the fact that intradermal transplants, which grew much slower, were firmer than tumors growing in other tissues.

TABLE 6.—*Relation of site of injection to ascorbic acid concentration of Crocker sarcoma 180 in strain C mice*

Site of injection	Number of animals	Ascorbic acid concentration per 100 gm. of fresh tissue		Average weight of tumor
		Range	Average	
		Milligrams	*Milligrams*	*Grams*
Intraperitoneal	9	9–31	19	1.8
Intramuscular	6	16–24	20	1.8
Subcutaneous	6	12–19	17	1.6
Intrahepatic	4	14–21	18	1.9
Intradermal	[1] 7	22–31	26	.25

[1] 2 duplicate analyses.

DISCUSSION

Biochemical characterization of neoplasms has shown that the concentration of the B vitamins is remarkably similar for all neoplasms (*29, 30*). The ascorbic acid concentration was not so constant, varying from 15 mg. per 100 gm. of tissue in a spindle-cell carcinoma of the salivary gland to 70 mg. per 100 gm. in leukemic lymph nodes. However, the variation of ascorbic acid in tumors was less than that between normal tissues. A comparison of the distribution of ascorbic acid concentrations in tumors and normal tissues is illustrated by the histograms in figure 2. A third histogram shows the distribution of one of the B vitamins, riboflavin, in tumors.

Since the concentration of ascorbic acid

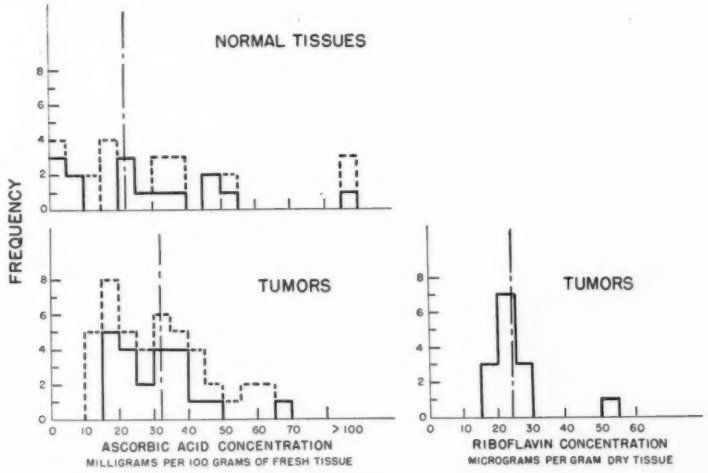

FIGURE 2.—Distribution of ascorbic acid concentrations between tumors and between normal tissues, and the distribution of riboflavin concentration between tumors. The frequency is the number of different tumors or normal tissues having an average concentration lying within the range indicated. The solid lines indicate data obtained by the author; the dotted lines include values taken from the literature. Dash-dot line represents the median.

in tumors varied over a wide range, an attempt was made to correlate it with some other properties of tumors. When the data were broken down into two groups, carcinomas and sarcomas, the ascorbic acid concentration exhibited the same variation within either group; there was no significant difference between the means of the two groups. The average concentration of ascorbic acid in 15 sarcomas reported in the literature and analyzed in the study was 29.9 mg. per 100 gm. (range 14 to 61) and of 12 carcinomas 32.7 mg. per 100 gm. (range 14 to 64).

The data show further that there was no correlation of the ascorbic acid concentration of the neoplasm with that of its tissue of origin. Thus, the tumor of the adrenal cortex, a neoplasm derived from a tissue containing a very high concentration of ascorbic acid, contained less of the vitamin than did mammary adenocarcinoma, rhabdomyosarcoma, or the fibrosarcomas, neoplasms derived from tissues having low concentrations of ascorbic acid. Further examination of this type to see whether tumors are generally higher or lower than the corresponding normal tissues showed that 7 of the tumors analyzed have a lower concentration and 11 a higher concentration than the tissue of origin and 4 about the same concentration.

Ascorbic acid concentration in normal tissues has been considered to bear a general physiologic relationship to rate of growth (*8*). However, this study suggested no similar relationship for neoplasms. Hepatoma 31 did not grow as fast as either regenerating or fetal liver but had a higher ascorbic acid concentration; the adrenal in the adult animal certainly grows much slower than the adrenal tumor, yet the vitamin C content was considerably higher. Even if rates of growth and ascorbic acid concentration in the same tumor type were compared, no correlation was evident. Four tumors in this study permitted this type of comparison, the tumor of the adrenal cortex, leukosis 72942, Crocker sarcoma 180, and the mouse hepatomas 587 and 98/15.

The tumor of the adrenal cortex grew more rapidly in successive transplants without undergoing histologic change. Thus the third generation transplant grew to an average size of 2 gm., the fourth generation transplant to 5 gm., and the fifth to 13 gm. in 2 months. The vitamin concentration was the same in all three groups. In the case of leukosis 72942, ascorbic acid increased with age of the tumor, but the rate of growth from the twelfth to the twentieth day was the same. No difference in ascorbic acid concentration of Crocker sarcoma 180 in young and old mice was observed although the tumor reached an average size of 2.6 gm. 12 days after transplantation into 2-month-old mice, whereas the size was only 1.2 gm. in 24-month-old mice.

An apparent rough correlation between rate of growth and ascorbic acid content was evident in the two mouse hepatomas, but in this case another difference between the two tumors might have been of more importance. Hepatoma 98/15 not only grew more slowly and contained less ascorbic acid than hepatoma 587 but invariably was more necrotic and contained large amounts of encysted blood. Edlbacher and Jung (*31*) and Woodward (*4*) showed that the necrotic portion of tumors contains very little ascorbic acid. Our experience fully corroborated this finding. Although all tumor specimens were freed as far as possible from necrotic material, small amounts might have remained and have been responsible for the observed small differences in ascorbic acid content.

From the preceding, it is evident that the ascorbic acid concentration of tumors was not found to be uniform, nor was the

variation related to differences in other tumor characteristics. The concentration of this vitamin seemed to be a property of the individual tumor and not affected by such factors as rate of growth, age of host, site of growth, or the ascorbic acid content of the parent tissue.

SUMMARY

The ascorbic acid content of 22 tumors and of comparable normal tissues was determined.

The ascorbic acid concentration of the tumors ranged from 15 to 70 mg. per 100 gm. of fresh tissue.

The ascorbic acid content of the tumors was not related to that of the tissues of origin.

No correlation between ascorbic acid concentration and rate of growth was evident.

Neither age of host nor site of transplantation materially affected the ascorbic acid concentration in the tumor.

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A Spontaneous, Transplantable, Adrenal Cortical Tumor Arising in a Strain C[1] Mouse

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Few spontaneous adrenal cortical tumors have been described as arising in laboratory animals; it is felt that a record of the present case might be of some interest.

The tumor was found in the right adrenal of a 24½-month-old strain C female mouse of the colony maintained at the National Cancer Institute and has been designated "adrenal tumor C–199." It was transplanted subcutaneously into two young strain C females on November 24, 1941, and was found positive in one May 1, 1942, and in the other on June 2, 1942. In succeeding generations the growth rate rapidly increased, so that in the fourth generation, transplants were found positive 5 days after transplantation. The tumor is now in the eleventh generation, and transplantation has been successful following all subcutaneous inoculations. While the number of animals involved is rather small, evidence is accumulating that a considerable difference in growth rate of the tumor exists which appears to be dependent on the sex of the host, the tumor growing more rapidly in males.

PATHOLOGY

PRIMARY TUMOR

At autopsy, the right adrenal of the original host was enlarged to 4 mm. and was of normal color. Gross metastases were not found. On histologic examination it was found that tumor tissue had replaced all of the medullary tissue and all except a narrow strip of cortex (fig. 1). The tumor was composed of closely packed cords of cells which showed a close association with the numerous capillaries of the tumor. These cords were usually two to four cells thick, and their outer limits were frequently demarcated by a delicate strand of reticulum which lay interposed between the epithelial cord on one side and the adjacent capillary on the other (fig. 2). The tumor cells showed a very indistinct cytoplasm and a relatively large, spherical or ovoid, heavily staining nucleus with a fairly prominent nuclear membrane. A considerable number of small chromatin granules were regularly present, either distributed evenly through the nucleus, or more often concentrated immediately beneath the nuclear membrane. One relatively large, prominent, basophilic nucleolus was characteristic. A few mitotic figures were noted.

The tumor had no capsule, and it was markedly compressing the remaining small amount of normal adrenal tissue and actually invading it as well.

[1] *Strain C mice are albino animals originally obtained from Dr. Snell, of the Roscoe B. Jackson Memorial Laboratory, in 1935 when they were in the* F_{35} *generation of inbreeding. This tumor arose in a mouse of the* F_{41} *generation of inbreeding.*

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Transplants

At autopsy the transplants were regularly found to possess an extraordinarily well developed blood supply, and section of the growth always gave evidence of its excessive vascularity by the rapid welling of blood from the cut surface. The tumor cells of the transplant appeared similar to the cells of the primary tumor with perhaps two exceptions—the cytoplasm appeared to stain more intensely and was eosinophilic. The cell membranes, while at times easily discernible, were often indistinct, thus producing a syncytium-like appearance of the tumor cords (fig. 3, *A*). Mitotic figures were much more numerous than in the primary tumor. While in certain areas of the transplant the cell cords were closely packed (fig. 3, *B*), in other areas (fig. 3, *A*) the epithelial cords lay parallel to each other and were separated by rather wide endothelial-lined sinusoids, recalling the pattern of the adrenal cortex and other endocrine glands. No rosette formations were found in any of the transplants or in the primary tumor. The tumor contained considerable reticulum in the form of varying-sized strands. The thicker fibers usually lay at the periphery of masses of epithelial cells thus emphasizing the distinct cord formation (fig. 4, *A*). There was some penetration of delicate fibers among individual tumor cells (fig. 4, *B*), but the fundamental picture of a cord arrangement was always maintained. While reticulum was regularly present, the phosphotungstic acid-haematoxylin stain failed to demonstrate any intracellular fibers in the tumor cells.

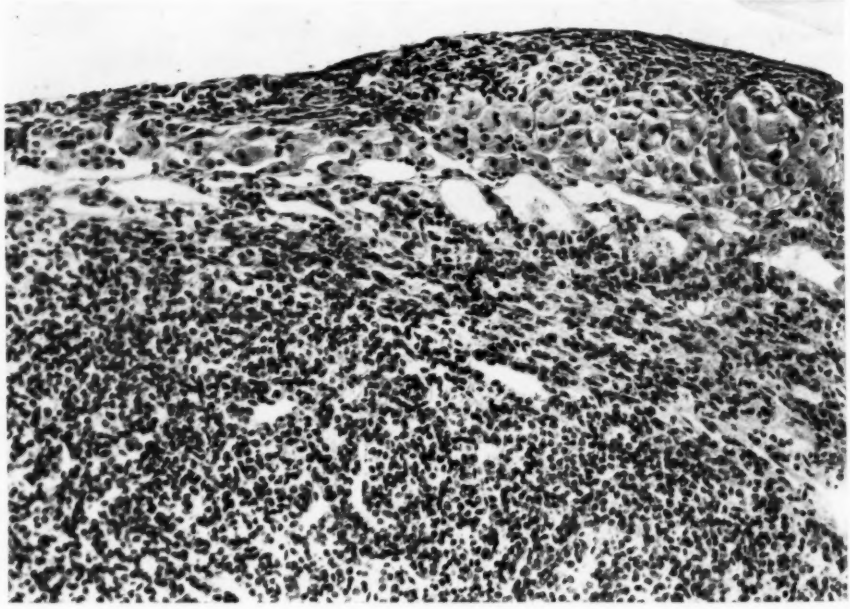

Figure 1.—Section of the primary tumor showing a small amount of normal cortical tissue in the upper part of the field. The small size of the tumor cells is obvious when compared with the normal cortical cells. Zenker-formol fixation, haematoxylin and eosin stain. × 160.

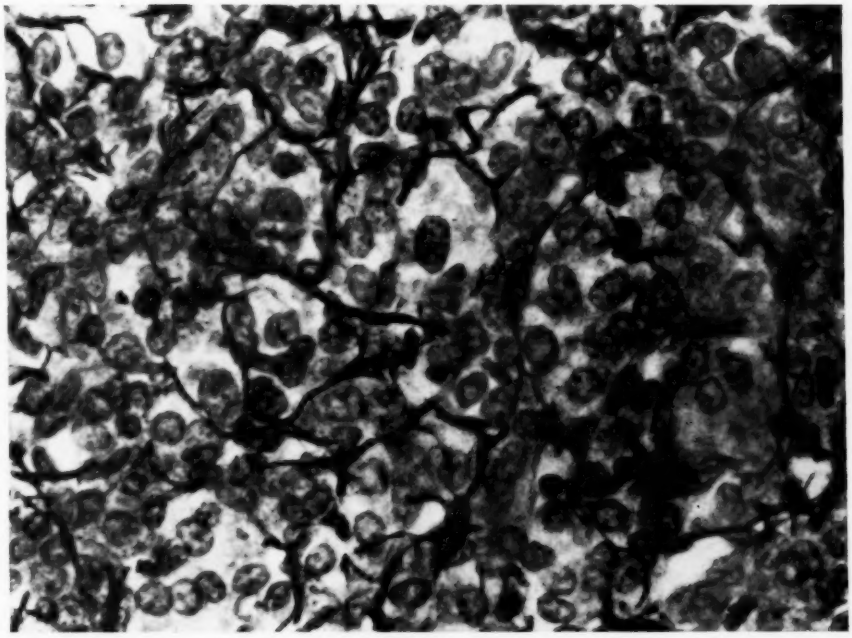

FIGURE 2.—Primary tumor showing the distribution of reticulum with coarser fibers surrounding groups of cells and finer fibers extending between individual cells.. Zenker-formol fixation and Laidlaw's stain for reticulum. × 465.

A definite capsule was regularly present around the transplant, but this capsule appeared to be continually invaded by tumor tissue, either diffusely (fig. 5) or in the form of distinct cords.

Necrosis was found regularly in the large tumors but to a lesser degree than, in our experience, is characteristic of the majority of transplantable neoplasms. In recently formed areas of necrosis an infarctlike process was apparent, while in the older areas most of the necrotic tumor tissue had been removed, and resulting inflammatory cells lay about the margin.

Stains for fat with sudan IV and osmic acid revealed the presence of small amounts of fat scattered in viable cells. Large amounts were found in areas of focal necrosis. In sections of tumor tissue from the first two generations of transplants treated with osmic acid, cells were frequently encountered which, because they contained osmiophilic droplets, appeared remarkably like the cells of the zona fasciculata of the normal adrenal glands (fig. 6, *A*, and *B*). Such cells have not been noted in later transplants, i. e., in transplants which have achieved a more rapid growth rate. Bearing in mind the systemic changes noted in animals with hyperplastic adrenal glands by Fekete, Woolley, and Little *(1)*, Woolley, Fekete, and Little *(2–4)*, and Gardner *(5)*, we carefully examined the host animals for gross and histologic changes, particularly in the urogenital system and in the adrenal glands. No changes were noted in the kidney, heart, blood vessels, liver, pancreas, spleen, lymph nodes, or lungs. However, in female mice focal edema was noted in the deeper

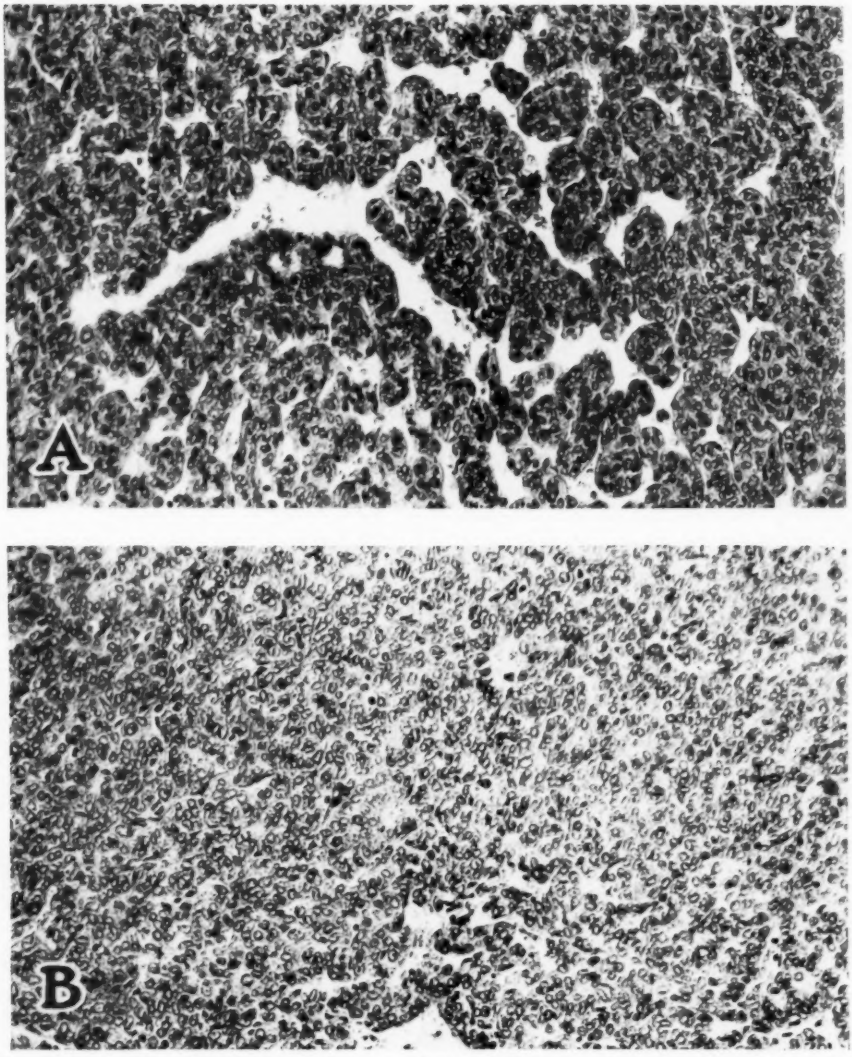

FIGURE 3.—Section of a first-generation transplant. *A*, Tumor cords separated by wide sinusoids; *B*, More solid areas of the tumor. Both *A* and *B*, Zenker-formol fixation and haematoxylin and eosin stain. × 225.

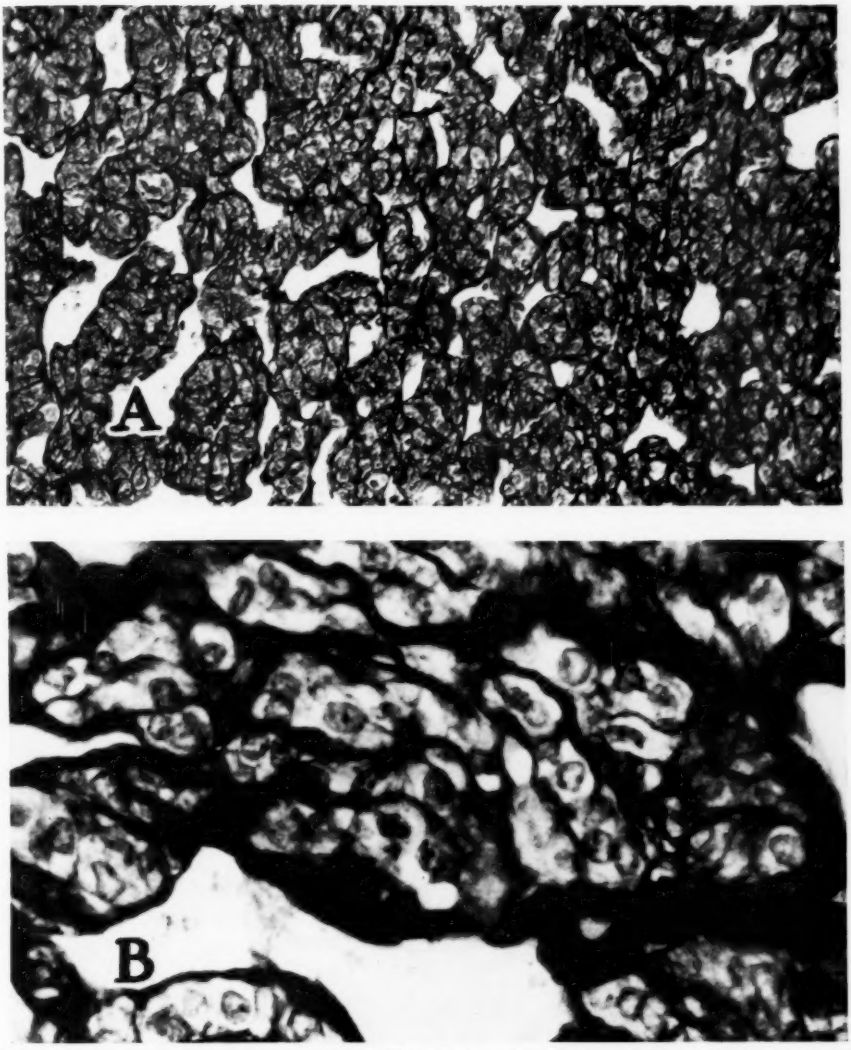

FIGURE 4.—First-generation transplant. *A*, Distribution of reticulum similar to that in the primary tumor. × 235; *B*, Distribution of the coarser reticulum fibers between cell cords and the neighboring sinusoids, and finer fibers between individual tumor cells. × 465. Both *A* and *B*, Zenker-formol fixation and Laidlaw's stain for reticulum.

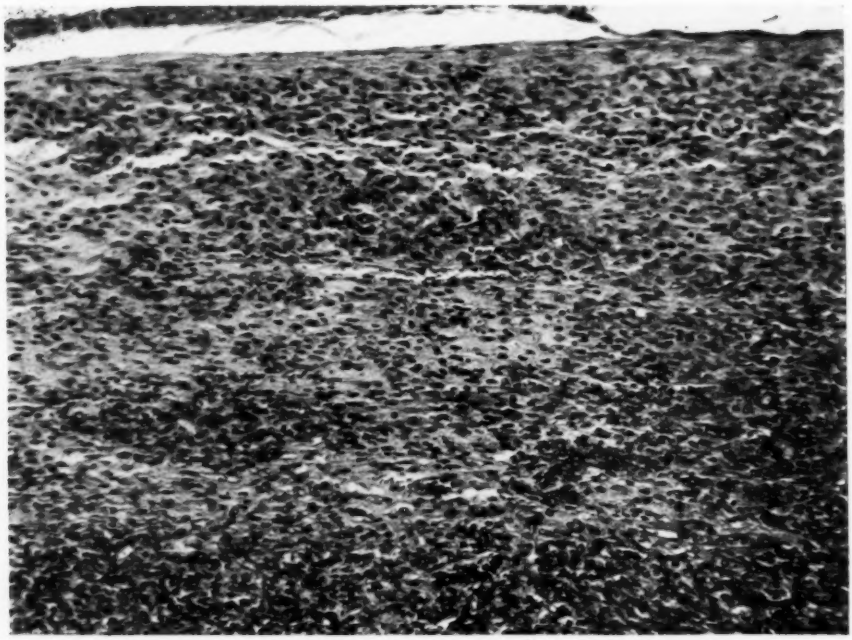

FIGURE 5.—Diffuse invasion of the capsule by tumor cells in a second-generation transplant. Zenker-formol fixation and haematoxylin and eosin stain. × 160.

layers of the endometrial stroma of the uterus. No changes were noted in the genital system of male hosts. Also the loss of lipid characteristic of the adrenal glands of tumor-bearing animals [2] was not noted in the adrenals of either male or female hosts bearing this tumor.

CYTOPATHOLOGY [3]

As indicated previously, the tumor cells possessed relatively little cytoplasm, and the cell boundaries were characteristically indistinct. The Golgi substance consisted of a small knot of osmiophilic material in close contact with the nuclear membrane (fig. 7, *A*). This type of arrangement of the Golgi material is not like that of any of the functional adrenal cortical cells as described by Bourne *(7)* and Dornfeld *(8, 9)*. It is similar, however, to that present in the cells transitional between capsule cells and true glomerulosa cells and is similar to the Golgi material of cells of the adrenal cortical adenomas occasionally found in the adrenals of old strain C female mice (fig. 7, *B*). The cells of these growths in some instances contain small amounts of lipoid (fig. 6, *B*).

Mitochondria of the capsule and transitional cells of the adrenal cortex have been found to be mostly filamentous in form, while those of the zona glomerulosa and zona fasciculata are mostly very small spheres with a few rods or filaments interspersed between them. The mitochondria of the tumor cells were somwhat variable in form. Those of the smallest cells were

[2] *Data to be published in this Journal.*
[3] *For details of the cytologic methods employed, see Dalton and Edwards* (6).

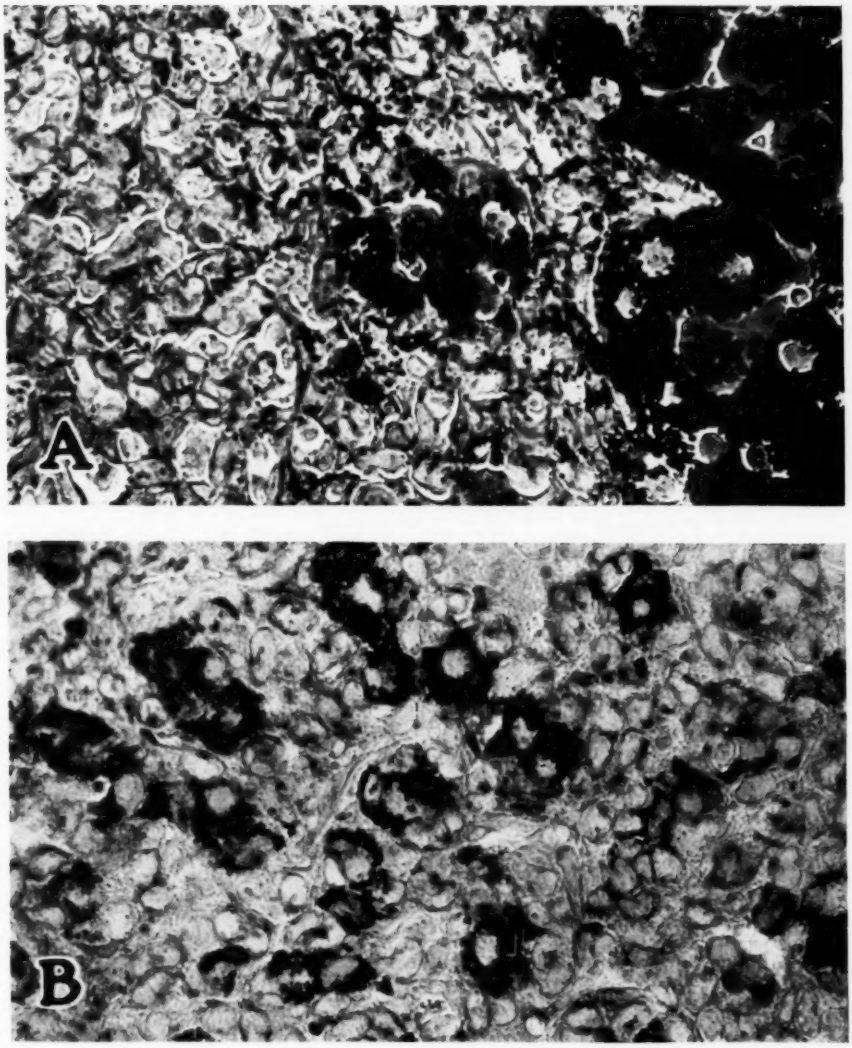

FIGURE 6.—*A*, Section of an adrenal of a 23-month-old strain C female mouse at the junction between an adrenal cortical adenoma and normal adrenal cortical tissue. The cells of the adenoma (left) contain few and scattered lipoid droplets compared with the normal fasciculata cells at the right. *B*, Section of first-generation transplant of the adrenal tumor showing scattered lipoid-filled cells similar to normal cortical cells in *A*. Both *A* and *B*, Champy fixation and osmic acid impregnation. × 1300.

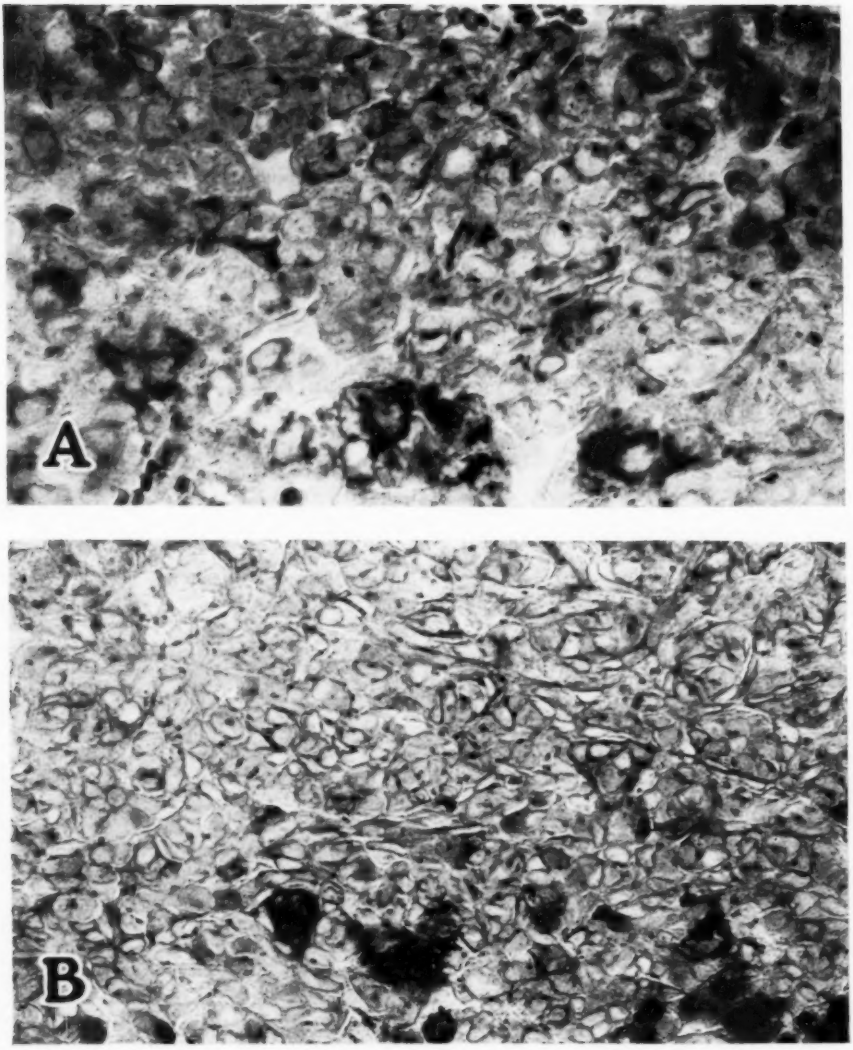

FIGURE 7.—*A*, First-generation transplant showing the small amount of juxtanuclear Golgi material characteristic of the tumor cells. Scattered lipoid-filled cells appear near the lower margin; *B*, Section of an adrenal of a 23-month-old strain C female mouse showing the Golgi material present in an adrenal cortical adenoma. The position and character of the Golgi material is similar to that of the adrenal tumor in *A*. Both *A* and *B*, Champy fixation and osmic acid impregnation. × 1300.

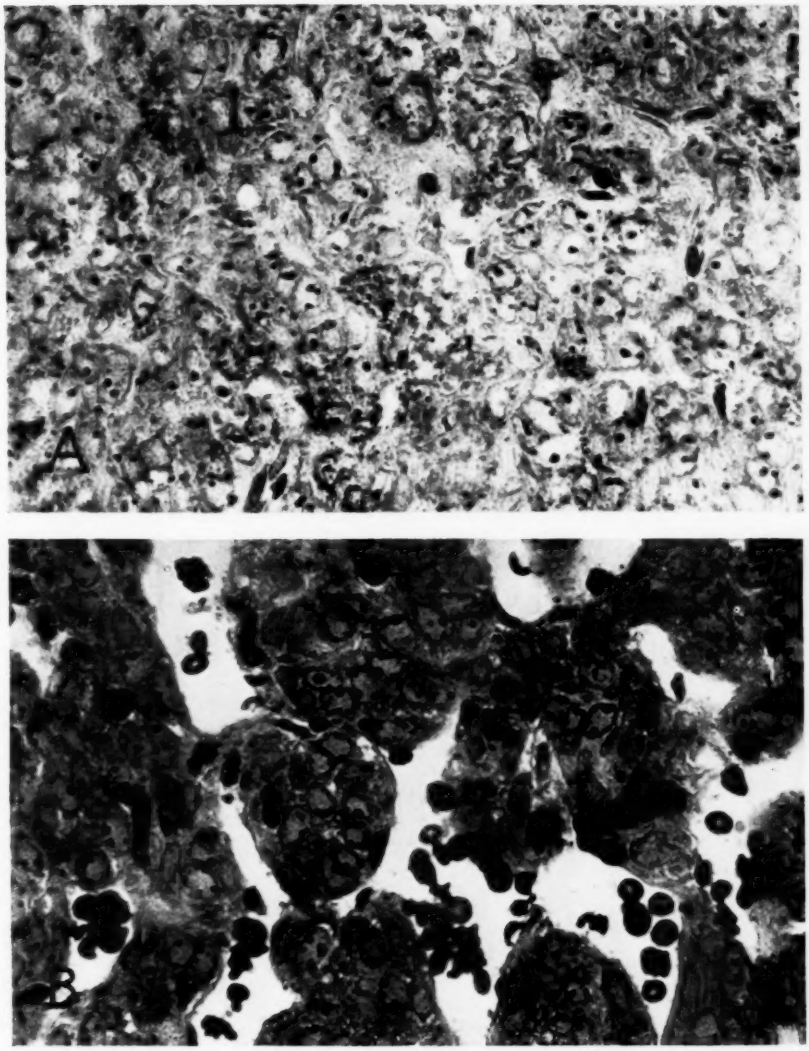

FIGURE 8.—*A*, Spherical mitochondria characteristic of the majority of the cells of the first-generation transplant of the adrenal tumor; *B*, Mitochondria of cells of the fourth-generation transplant. They are very thin, tenuous filaments. Both *A* and *B*, Regaud fixation and Heidenhain's iron haematoxylin stain. × 1300.

filamentous, while in the cells with larger amounts of cytoplasm and in cells containing lipoid droplets, they were predominantly spherical. In general, larger cells containing spherical mitochondria were characteristic of the first two generations of transplants (fig. 8, *A*) and smaller cells with filamentous mitochondria have been found to be characteristic of later generations (fig. 8, *B*).

DISCUSSION

The general architecture and the histologic and cytologic evidence support the view that the tumor arose originally from relatively undifferentiated cells of the adrenal cortex. The work of Salmon and Zwemer *(10)* indicates that such cells exist in the normal gland since they have shown that new functional cells are continually being formed as a result of the differentiation of the inner capsule cells. The spontaneous adenomas described herein and the nodular hyperplasias described by Woolley, Fekete, and Little regularly arose in a subcapsular position. Gardner stated that the origin of the hyperplastic cortical tissues in his studies could not be determined accurately but that they appeared to originate in the zona glomerulosa. It is suggested that these adenomas and the adrenal tumor C 199 originated in the same region and from similar cells.

The failure to find any evidence of metastases in the original host and in the hosts of later generations of transplants suggested that the neoplasm was relatively benign in character. However, the rapid growth rate attained in later generations plus the evidence of invasion of the capsule by the tumor cells indicated a certain degree of malignancy. For these reasons the tumor has been tentatively diagnosed as a carcinoma of the adrenal cortex of low grade malignancy.

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